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Isolation and characterisation of an *Hsp90* homologue from the resurrection plant *Xerophyta viscosa*

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A dissertation submitted in fulfillment of the requirements for the degree of Master of Science in the Department of Microbiology, Faculty of Science, University of Cape Town, South Africa

February 2002

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ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisors, Dr. Sagadevan Mundree, Prof. Jennifer Thomson and Assoc. Prof. Jill Farrant, for their invaluable advice, guidance and encouragement during the course of my studies. I would also like to thank the members of my lab, Dahlia, Shaheen, Shaun, Saberi, Kershini, Jonathan, Nyaradzo, Marion, Yasemin, Sam and Bienyameen, for their company, willingness to help and moral support when things were not going well. To Di James, a big thank-you for help with DNA sequencing and to Anne Marie Clennel and Di De Villiers for always making sure enzymes and reagents were available. We wouldn't be able to do it without you. Also thank-you to the plant physiologists, Clare van der Willigen, Keren Cooper and Debbie Loffell for their advice during the course of this project.

Thank-you to my parents, Derek and Celia Walford, for their love, support and guidance, I owe you more than I can say. And finally to Keith Quixley, without whom I would be lost, a huge thank-you for all the support, encouragement and guidance, as well as all the proofreading.

I acknowledge the financial support provided by the NRF and the Microbiology Department.

ABBREVIATIONS

ABA	abscisic acid
ADP	adenine diphosphate
Amp	ampicilin
Amp^R	ampicillin resistance
ATP	adenine triphosphate
Aw	water activity
Bp	base pair(s)
BiP	binding protein
Ca^{2+}	calcium
cDNA	copy DNA
DEPC	diethylpyrocarbonate
dH_2O	distilled water
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
EDTA	ethylenediaminetetra-acetic acid
g	grams
HCl	hydrochloric acid
H_2O	water
IP_3	inositol-(1,4,5)triphosphate
IPTG	isopropyl-β-D-thiogalactopyranoside
kB	kilobase(s)
KCl	potassium chloride
kDa	kilodalton(s)
λ	lambda
l	litre(s)
LA	Luria-Bertani agar
LB	Luria-Bertani broth
M	molar concentration
µg	microgram(s)
mg	milligram(s)
$MgSO_4$	magnesium sulphate
µl	microlitre(s)
ml	millilitre(s)
µm	micrometer(s)
mM	millimolar
min	minutes
mRNA	messenger RNA
mW	molarity of water
NaAc	sodium acetate
NaCl	sodium chloride
NaOH	sodium hydroxide
Ni-NTA	nickel-nitrilotriacetic acid
nm	nanometre(s)
nt	nucleotide(s)
OD	optical density

ORF	open reading frame
PMSF	phenylmethanesulfonylflouride
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
RWC	relative water content
SDS	sodium dodecyl sulphate
Spp	species
S6K	ribosomal S6 protein kinase
TBE	tris borate EDTA
TE	tris-EDTA
Tris	tris(hydroxymethyl)aminomethane
u	unit(s) of enzymatic activity
UV	ultraviolet
w/v	weight per volume
WP	water potential
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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ABSTRACT

Prior to this study, a cDNA library of dehydrated *Xerophyta viscosa* was differentially screened and several genes were found to be upregulated during dehydration. One of these cDNAs was found to share a high degree of sequence identity with the ER-located Hsp90 or Grp94 family of proteins (hereafter referred to as *XVGrp94*) and forms the basis of this work. The *XVGrp94* cDNA was found to be truncated at the 5` terminus and a full length cDNA was isolated using SMART-RACE™ (Switching Mechanism at 5` end of RNA Transcript – Random Amplification of Complementary Ends). This cDNA was sequenced and appeared to be a representative of the *Hsp90* family of genes. The putative gene contained an ORF (Open Reading Frame) potentially coding for an 812 amino acid protein with a calculated size of 92.83 kDa. It shares 85% homology with other Hsp90s from plants and it contains several characteristic features of these proteins. Additionally, it contains the ER (endoplasmic reticulum) targeting and retention signals. Southern blot analysis confirmed the presence of the gene in the *X. viscosa* genome possibly as a member of a family of closely related genes. Northern blot analysis revealed a transcript size of 2.8 kb, however, expression patterns of the transcript could not be established. Western blot analysis showed that the XVGrp94 concentration increased significantly in response to heat and dehydration, and a slight increase was observed in response to conditions of high salt, but no response was seen in response to high light, cold or exogenous ABA (abscisic acid) application. The *XVGrp94* open reading frame was cloned into the pProEX HTa expression vector and expressed in *E. coli*, but purification of the recombinant protein was not successful.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1. Water deficit

The ability of a plant to respond to constantly changing environments determines its survival. One of the major challenges a plant has to withstand is water deficit, caused by drought, high salinity or low temperature (Bohnert *et al.,* 1995). Water is vital for plant survival. It is a solvent for biochemical reactions and it stabilises structures of molecules and organelles through hydrophilic and hydrophobic interactions (Koster and Leopold, 1988, Jones, 1992). Water is also involved in processes such as photosynthesis and hydrolysis, while its thermal properties allow it to act as an evaporative coolant and its incompressibility is important in support and growth (Jones, 1992). Plant water deficit, which occurs when the rate of transpiration exceeds that of water uptake (Bray, 1997), results in damage to the cell membranes and can disrupt general cellular organisation. Growth and metabolism cease and the plant may eventually die (Furini *et al.,* 1997).

1.2. Resurrection plants

Many plants are able to withstand minor fluctuations in water availability, but very few are able to survive extreme conditions (Bewley and Oliver, 1992; Sherwin and Farrant, 1995; Ingram and Bartels, 1996). A small number of plants, referred to as desiccation-tolerant or resurrection plants, have developed mechanisms to allow them to tolerate extreme desiccation. They are able to tolerate water loss of greater than 90% and are able to recover quickly upon rehydration (Gaff, 1971; Bewley and Oliver, 1992; Vertucci and Farrant, 1995; Ingram and Bartels, 1996).

Most resurrection plants, not surprisingly, are native to arid climates of the world and are found in southern Africa, southern America and western Australia (Gaff, 1977, 1987). Many grow in shallow soils on rocky outcrops where there is little shade and the water supply is limited (Sherwin and Farrant, 1995). These shallow soils are quickly, but temporarily, flooded when it rains and the water only lasts a few days at best. The growth and reproduction of these plants occurs during the wet period, but upon drying the plants are able to remain in a dormant state for lengthy periods (Scott, 2000).

Desiccation tolerant plants are categorised as either fully-desiccation tolerant, or modified-desiccation tolerant (Bewley and Oliver, 1992; Oliver and Bewley, 1997; Oliver *et al.*, 1998). Fully-desiccation tolerant plants are found among the less complex plants including algae, bryophytes and lichens (Bewley and Oliver, 1992). These plants are unable to retard water loss and as a result their internal water content readily equilibrates to that of the environment. As a consequence these plants can experience extreme drying rates and may reach air dryness within an hour (Bewley and Oliver, 1992; Oliver and Bewley, 1997; Oliver *et al.*, 1998). The majority of modified-desiccation tolerant plants are members of the more complex plant groups from ferns and angiosperms (Bewley and Oliver, 1992; Oliver and Bewley, 1997). These plants survive only when drying occurs over several hours or days. This allows time for protective measures to be induced and the establishment of tolerance before the plant dries completely (Bewley and Oliver, 1992; Oliver and Bewley, 1997; Farrant *et al.*, 1999).

The monocotyledonous plant, *Xerophyta viscosa* Baker (Family Velloziaceae) (Figure 1.1) is an example of such a resurrection angiosperm (Gaff, 1971). *X. viscosa* dehydrates slowly to a relative water content (RWC) of 5% and upon rewatering the plant rehydrates completely and is able to resume full physiological activities within 80 hours (Sherwin and Farrant, 1996).

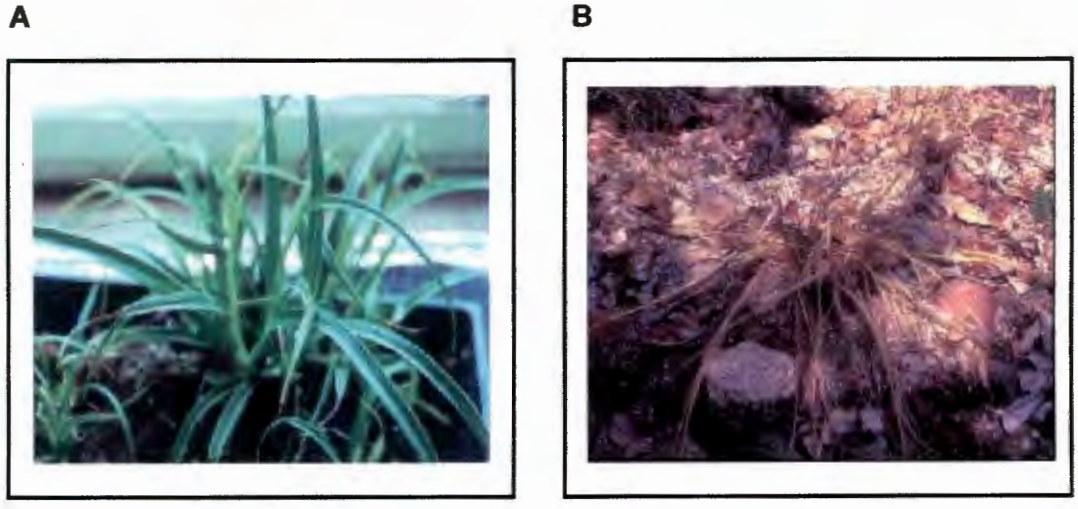

Figure 1.1: *Xerophyta viscosa*, a resurrection angiosperm.
A) An example of *X. viscosa* in its fully hydrated state. B) An example of fully dehydrated *X. viscosa* on a rocky outcrop. Note the brown colour of the leaves, indicating the loss of chlorophyll, and accumulation of anthocyanins, during desiccation.

1.2.1. Mechanisms of desiccation tolerance

The mechanisms of desiccation tolerance in lower order resurrection plants is different from that present in angiosperms (Oliver and Bewley, 1997; Oliver *et al.,* 1998), and as crop plants are angiosperms, the mechanisms employed by this group of plants to survive are of relevance to the current study. Although the mechanisms allowing these plants to survive desiccation are far from completely understood, it is becoming clear that resurrection angiosperms achieve tolerance through the induction of protective mechanisms during dehydration. These operate together with repair of any damage that may have occurred during dehydration or rehydration (Ingram and Bartels, 1996; Oliver and Bewley, 1997; Farrant and Sherwin, 1996, Sherwin and Farrant, 1998; Oliver *et al.,* 1998; Farrant, 2000; Farrant and Cooper, 2002).

In order to survive resurrection plants must overcome a number of subcellular stresses caused by drying (Bewley and Oliver, 1992; Oliver and Bewley, 1997; Vertucci and Farrant, 1995). These include: 1) mechanical stresses associated with turgor loss as a consequence of volume reduction. The stress arises as the large central vacuole shrinks and cell contents are drawn inwards, creating tension between the plasmalemma and the rigid cell wall. Rupturing of the plasmalemma results in cell death (Iljin, 1957). 2) The disruption or loss of macromolecular integrity, occurring as a result of the increase in protein and ion concentration during water deficit. 3) Free radical-mediated damage resulting from unregulated metabolism. These reactive oxygen species can have deleterious effects on membranes, proteins and DNA (McAinsh *et al.,* 1996).

In angiosperms the protective mechanisms against desiccation damage are complex and also vary among species (Farrant and Sherwin, 1998, Farrant, 2000). These mechanisms include: 1) the minimisation of mechanical damage associated with tugor loss by subcellular re-organisation, such as cell wall folding as seen in *Craterostigma* spp and replacement of water with non-aqueous substances in vacuoles, as seen in *Xerophyta* spp (Farrant and Sherwin, 1998; Vicre *et al.,* 1999; Farrant, 2000). 2) The production of proteins, sugars and compatible solutes which replace water and maintain macromolecular and membrane integrity and stabilise the cellular environment. This also has a role in maintaining cellular volume and thus relieves mechanical stress due to turgor loss (Bianchi *et al.,* 1993; Ingram and Bartels, 1996; Oliver and Bewley, 1997; Oliver *et al.,* 1998; Scott, 2000; Hoekstra *et al.,* 2001). 3) Antioxidant production to scavenge free radicals produced by unregulated metabolism (Sherwin and Farrant, 1998; Farrant, 2000). 4) Mechanisms to avoid light damage, as light energy trapped by chlorophyll cannot be dissipated via photosynthesis

during dehydration. The energised electrons, which may in turn generate free oxygen radicals can cause considerable subcellular damage (Smirnoff, 1993). In addition to the production of antioxidants, resurrection plants also utilise a mechanism to physically prevent free radical formation. Poikilochlorophyllous plants, such as *X. viscosa,* avoid light damage by losing chlorophyll and dismantling thylakoid membranes during dehydration (Tuba et al., 1993, 1994, 1996; Sherwin and Farrant, 1998). The photosynthetic apparatus has to be reconstituted upon rehydration and thus results in a delay in recovery of carbon metabolism. Homoiochlorophyllous species retain chlorophyll but fold their leaves to shade chlorophyll or produce pigments such as anthocyanin or carotenoid to prevent/or reduce the damage associated with light (Tuba *et al.,* 1993, 1994, 1996; Sherwin and Farrant, 1996, 1998, Farrant, 2000).

1.2.2. Resurrection plants as models for studying desiccation tolerance

Resurrection plants provide excellent models to study water deficit tolerance at the physiological, genetic and biochemical levels and are being studied to further our understanding of plant responses to water deficit. These plants are a potential source of genes to enhance stress tolerance. The underlying mechanisms together with the genes involved in desiccation tolerance can be exploited to improve the drought tolerance of drought-sensitive crops.

As can be seen from the study of plant desiccation tolerance one specific mechanism does not confer tolerance on its own, but the interplay of several mechanisms simultaneously is required. This requires an integrated response at the whole plant level as well as a cellular response. To understand the mechanisms by which a plant may survive desiccation, it is necessary to know how plant cells sense the loss of water; how the stress signals are transduced into cellular signals and transmitted to the nucleus; how gene transcription is affected by these stress signals and, finally, how the gene products function in stress tolerance (Shinozaki and Yamaguchi-Shinozaki, 1996).

1.3. Regulation of gene expression during dehydration

Plants respond to dehydration at the cellular and molecular levels. Drought stress induces the expression of various genes that are involved in the stress response and tolerance (Bohnert *et al.,* 1995; Ingram and Bartels, 1996; Bray, 1997; Shinozaki and Yamaguchi-Shinozaki, 1997; Shinozaki *et al.,* 1999). Studies have begun to elucidate mechanisms that

control gene expression and putative regulatory pathways have been established. However, the current knowledge of these pathways is incomplete and most of the studies have used *Arabidopsis thaliana* as a model plant (Shinozaki and Yamaguchi-Shinozaki, 1997; Shinozaki *et al.,* 1999). The resurrection plant *Craterostigma plantagineum* has also been studied fairly extensively (Ingram and Bartels, 1996). Very little is known about signal perception, whereas signal transduction pathways are beginning to be understood. In addition *cis* acting control elements of the promoters of several of the response genes have been identified and characterised (Ingram and Bartels, 1996; Bray, 1997; Shinozaki and Yamaguchi-Shinozaki, 1997; Shinozaki *et al.,* 1999).

1.3.1. Signal perception and transduction

The regulation of the water deficit response initially requires the recognition of the stress. This can then trigger a cellular signal transduction pathway, converting a physical response into a biochemical response. Not much is known about how a plant cell perceives water stress, but possible sources of information such as change in osmotic potential across the plasma membrane due to a decrease in turgor pressure, change in cell volume or membrane area, loss of membrane flexibility, change in solute concentration and changes in cell wall plasma membrane connections may signal water deficit to the cell (Bray, 1997). It has been suggested that a change in osmotic pressure is a major trigger of the dehydration response at the molecular level (Shinozaki and Yamaguchi-Shinozaki 1997). Two-component systems appear to function in sensing osmotic stress in plants as well as in bacteria and yeast (Mizoguchi *et al.,* 1997; Wurgler-Murphy and Saito, 1997). A two-component histidine kinase (ATHK1) has recently been isolated from *Arabidopsis* and has been shown to function as an osmosensor in yeast (Urao *et al.,* 1999). ATHK1 has a typical histidine kinase domain, a receiver domain and two transmembrane domains in the amino terminal domain and has been proposed to function in signal perception during dehydration stress in *Arabidopsis.* Response regulators (Urao *et al.,* 1998) and phospho-relay intermediators (Miyata *et al.,* 1998) have also been isolated from *Arabidopsis*, suggesting that plants have an osmosensing and signaling system similar to that of yeast (Shinozaki *et al.,* 1999). This is probably not the only sensing mechanism functioning during drought stress responses, and other sensing mechanisms such as mechanical sensors of cytoskeletons and sensors of superoxides produced by stress may also have a role (Figure 1.2) (Shinozaki *et al.,* 1999).

An upregulation of genes encoding proteins involved in signal transduction cascades has been observed in plants in response to environmental stresses such as dehydration, cold

and high salinity (Shinozaki *et al.*, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000). These include, mitogen-activated protein (MAP) kinases, calcium-dependent protein kinases (CDPK) as well as enzymes involved in phospholipid metabolism, such as phospholipase C (PLC), phospholipase D (PLD), phosphatidylinositol-4-phosphate-5-kinase (PIP5K) and inositol-(1,4,5)triphosphate (IP_3) (Hirayama *et al.*, 1995; Jonak *et al.*, 1996; Mizoguchi *et al.*, 1997; Mikami *et al.*, 1998; Bjorn *et al.*, 2000; Frank *et al.*, 2000; Kiegerl *et al.*, 2000). These signaling factors are possibly involved in the amplification of signals in response to osmotic stress (Figure 1.2) (Shinozaki *et al.*, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000).

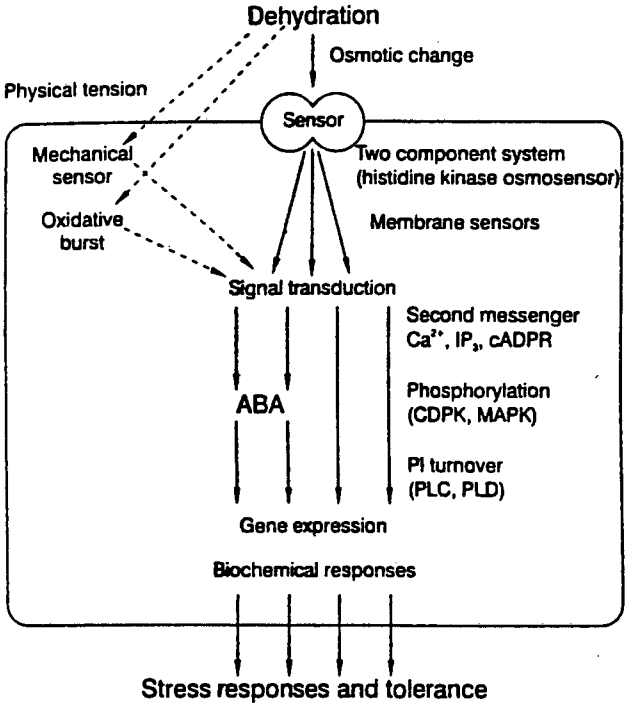

Figure 1.2: Second messengers and factors involved in the signal perception and signal transduction in the drought stress response. A two-component histidine kinase is thought to function as an osmosensor in plants. Ca^{2+} and IP_3 (inositol-3-phosphate) are most probably second messengers in the dehydration signaling pathway. The phosphorylation cascade functions in both the ABA (abscisic acid) and water deficit signal transduction pathways. Phosphatidylinositol (PI) is also implicated in the pathway. CDPK, calcium-dependent protein kinase; MAPK, mitogen activated protein kinase; PLC, phospholipase C; PLD, phospholipase D. (Shinozaki *et al.*, 1999).

Stomata closure is a well-characterised response of plants to dehydration stress and ABA treatment (Knight *et al.*, 1997; Shinozaki and Yamaguchi-Shinozaki, 1997; Knight and Knight, 1999). During stomata closure cytoplasmic Ca^{2+} levels increase and it has been suggested that Ca^{2+} functions as a second messenger in the osmotic stress response (Figure 1.2) (Knight *et al.*, 1997; Knight and Knight, 1999; Shinozaki *et al.*, 1999). IP_3 (inositol-3-phosphate) and Ca^{2+} are probable candidates for second messengers in plant cells (Knight and Knight, 1999; Shinozaki *et al.*, 1999). In animal cells PIP5K catalyses the

production of phosphatidylinositol-4,5-bisphosphate from phosphatidylinositol-4-phosphate. PIP_2 is digested by PLC to produce two second messengers IP_3 and diacylglycerol. IP_3 then induces Ca^{2+} release into the cytoplasm which, in turn causes various responses in the cytoplasm. A similar system has been proposed for plants during water deficit stress (Knight and Knight, 1999; Shinozaki *et al.,* 1999).

ABA has an important role in drought stress response and is involved in stomata closure and the induction of many genes (Bray, 1997; Shinozaki and Yamaguchi-Shinozaki, 1997; Shinozaki *et al.,* 1999). Analysis of ABA signaling mutants has revealed that protein dephosphorylation and protein farnesylation are involved in ABA signaling (Shinozaki *et al.,* 1999). Various signaling molecules, such as phosphatidic acid and cyclic ADP ribose, as well as various protein kinases and enzymes involved in phospholipid metabolism also appear to be involved in ABA signaling. MAPK cascades and CDPK have also been suggested to have a role in ABA signaling pathways (Shinozaki *et al.,* 1999).

1.3.2. Complex regulatory networks of gene expression by desiccation stress

Following cellular perception of water loss, a signaling mechanism must be activated to induce specific genes. Analysis of expression of desiccation responsive genes have shown various patterns of induction with respect to timing as well as the imposed stress (Shinozaki *et al.,* 1999).

ABA, the plant growth regulator, is produced under conditions of cold, drought and high salinity and is important in mediating the physiological, cellular and molecular responses of plants to these stresses (Ingram and Bartels, 1996; Bray, 1997, Shinozaki and Yamaguchi-Shinozaki, 1997; Shinozaki *et al.,* 1999). It appears that dehydration causes the production of ABA, which then induces the expression of various genes (Shinozaki *et al.,* 1999). Genes that are positively or negatively regulated by ABA and/or water stress have been studied (Ingram and Bartels, 1996; Bray, 1997; Shinozaki and Yamaguchi-Shinozaki, 1997; Shinozaki *et al.,* 1999), and it has been shown that most, but not all stress inducible genes, require ABA under drought, salinity or cold stress. For example, ABA-deficient mutants were used to analyze drought inducible genes that respond to ABA. Several genes were induced by exogenous ABA treatment, but it was also found that cold or drought could induce expression of these genes in ABA-deficient (*aba*) or ABA-insensitive (*abi*) *Arabidosis* mutants (Nordin, *et al.,* 1991; Gilmour and Thomashow, 1991; Yamaguchi-Shinozaki and Shinozaki, 1993). These observations indicate that although these genes do not require an

accumulation of endogenous ABA under cold or drought conditions, they do respond to ABA. These results indicate that there are ABA-independent as well as ABA-dependent pathways to regulate the expression of genes under osmotic stress (Shinozaki and Yamaguchi-Shinozaki, 1997; Shinozaki *et al.*, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000).

As shown in Figure 1.3, it has been proposed that there are at least four independent signal pathways functioning in the activation of stress-inducible genes under conditions of osmotic stress. Two of these are ABA dependent (pathways I and II) and two are ABA independent (pathways III and IV) (Shinozaki and Yamaguchi-Shinozaki, 1997; Shinozaki *et al.*, 1999).

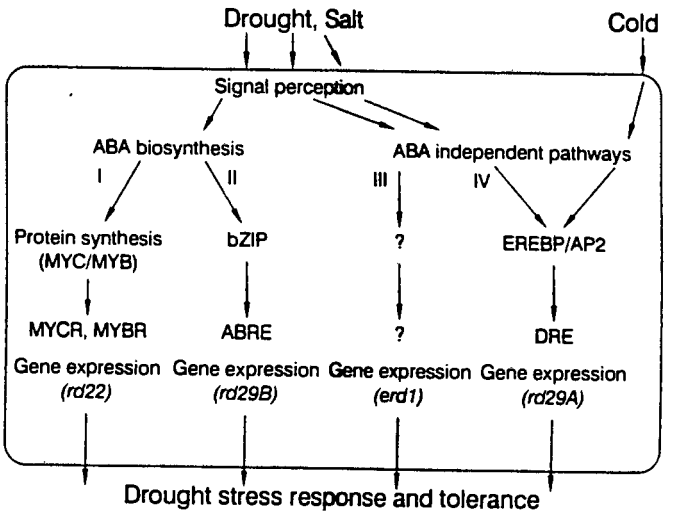

Figure 1.3: Signal transduction pathways linking the perception of stress signal and gene expression. At least four signal transduction pathways exist (I-IV): two are abscisic acid (ABA)- dependent (I and II) and two are ABA-independent (III and IV). Protein biosynthesis is required in one of the ABA-dependent pathways (I). In another ABA-dependent pathway, ABA-responsive element (ABRE) does not require protein biosynthesis (II). In one of the ABA-independent pathways, a drought responsive element (DRE) is involved in the regulation of genes not only by drought and salt, but also by cold stress (IV). Another ABA-independent pathway is controlled by drought and salt, but not by cold (III). (Shinozaki and Yamaguchi, 1997).

The first two pathways include ABA–dependent gene expression (pathway I) and ABA responsive gene expression (pathway II). In pathway I the production of protein factors is required to induce the expression of water-stress-inducible genes. The promoters of these genes have no ABREs (ABA responsive elements) but do contain several conserved DNA-binding motifs to which transcription factors such as MYC (from Myelocytoma, a virus induced tumour) and MYB (from Myeloblastoma) bind (Shinozaki and Yamaguchi-Shinozaki, 1997). Genes induced by dehydration and ABA have been found recently that encode transcription factors homologous to MYC and MYB (Iturriaga *et al.*, 1996; Urao *et al.*, 1993, Abe *et al.*, 1997). It is suggested that these transcription factors may function to regulate

ABA-inducible genes, such as *rd*22 in *Arabidopsis* (Yamaguchi-Shinozaki and Shinozaki, 1993), which respond to dehydration slowly, after the production of these transcription factors. In pathway II water stress inducible genes are upregulated by ABA and preceding protein biosynthesis (such as transcription factors). These ABA-inducible genes contain potential ABREs in their promoter region (in section 1.3.3). The ABRE contains motifs for bZIP protein binding. cDNAs encoding DNA-binding proteins that bind specifically to ABREs have been cloned and they have been found to contain bZIP structures, however, but it is unclear how ABA activates these bZIP proteins to bind to the ABRE and initiate the transcription of ABA-inducible genes (Shinozaki and Yamaguchi-Shinozaki, 1997; Shinozaki *et al.,* 1999).

Pathway III, which is ABA-independent is regulated by drought and salt, but not cold (Shinozaki and Yamaguchi-Shinozaki, 1997). Very little is known about this pathway. Two genes, *rd*19 and *rd*29 encoding different cysteine proteases (Koizumi *et al.,* 1993), and *erd*1 encoding a Clp protease regulatory subunit (Kiyosue *et al.,* 1993; Nakashima *et al.,* 1997) from *Arabidopsis* are some of the genes found to be regulated via this pathway. Analysis of their promoters may reveal more information about this pathway (Shinozaki and Yamaguchi-Shinozaki, 1997; Shinozaki *et al.,* 1999).

The other ABA-independent pathway (pathway IV) overlaps with that of the cold response pathway. A DRE (drought responsive element) is involved in the regulation of genes by drought and salt as well as cold (Shinozaki and Yamaguchi –Shinozaki, 1997; Shinozaki *et al.,* 1999). This will be discussed further in section 1.3.3.

Genetic analysis of *A. thaliana* mutants transformed with a *rd*29A promoter:luciferase reporter gene has indicated the presence of complex drought-, salt-, and cold-stress responses (Ishitani *et al.,* 1997). It has been proposed that ABA- and stress signaling pathways are not independent and that the various stress signaling pathways (including ABA-dependent and ABA-independent) are not completely independent. This suggests that there is a degree of cross-talk between the different pathways and this is further evidence for the complexity of regulation of the stress response in plants.

1.3.3. Importance of *cis*- and *trans*- acting elements

To understand the molecular mechanisms of gene expression in response to osmotic stress, *cis*- and *trans*-acting elements that function in ABA-responsive and in ABA-independent gene expression by drought stress have been analysed (Bray, 1997; Yamaguchi-Shinozaki,

1994). Two classes of DNA elements have been identified so far, the dehydration response element (DRE) and the ABA response element (ABRE) (Bray, 1997, Yamaguchi-Shinozaki and Shinozaki, 1994; Shinozaki and Yamagcuhi-Shinozaki, 1997; Shinozaki *et al.*, 1999).

Drought Response Element

Elements within the promoter region are important in determining the regulation of the gene under different conditions. A 9-bp conserved sequence TACCGACAT, named the DRE/CRT (dehydration responsive element or C-repeat) has been implicated in both dehydration and low-temperature responsive gene expression (Yamaguchi-Shinozaki and Shinozaki, 1994; Wang *et al.,* 1995; Iwasaki *et al.,* 1997; Stockinger *et al.,* 1997; Liu *et al.,* 1998; Thomashow; 1998; Shinozaki and Yamaguchi-Shinozaki, 2000).

Recently, five cDNAs encoding DRE-binding proteins (DREB) have been cloned from *A. thaliana* using the yeast one hybrid screening system (Liu *et al.,* 1998). All five are classified into two groups, DREB1 and DREB2. There are three DREB1 proteins (DREB1A, DREB1B, DREB1C) and two DREB2 proteins (DREB2A, DREB2B). Prior to this, a DREB-like protein was isolated from *Arabidopsis* and has been called CBF1 (CRT-binding factor) (Stockinger *et al.,* 1997). Subsequently two CBF1 homologues have been isolated, namely CBF2 and CBF3 (Gilmour *et al.,* 1998). Both DREB1A and DREB2A bind specifically to DRE/CRT and function as transcriptional activators (Liu *et al.,* 1998). Interestingly transcripts encoding DREB/CBF-like proteins have also been identified in *Brassica napus*, and were shown to induce expression of DRE-containing genes and they have also been identified in wheat, rye and tomato (Jaglo, *et al.,* 2001).

The expression pattern of the DREB genes has been analysed, and expression of DREB1A/CBF3 and its two homologues (DREB1B/CBF1 and DREB1C/CBF2) is induced by low temperature stress, while expression of the two DREB2 genes is induced by dehydration (Liu *et al.,* 1998; Gilmour *et al.,* 1998). These results suggest that DREB1 proteins are involved in cold-specific gene expression, whereas DREB2 proteins have a role in dehydration-specific gene expression (Liu *et al.,* 1998; Gilmour *et al.,* 1998; Shinozaki *et al.,* 1999; Shinozaki-Yamaguchi, 2000). These transactivators reveal how a gene can respond to different environmental stresses via two different signal transduction pathways in an ABA-independent manner (Figure 1.4) (Liu *et al.,* 1998; Shinozaki *et al.,* 1999; Shinozaki and Yamaguchi-Shinozaki, 2000).

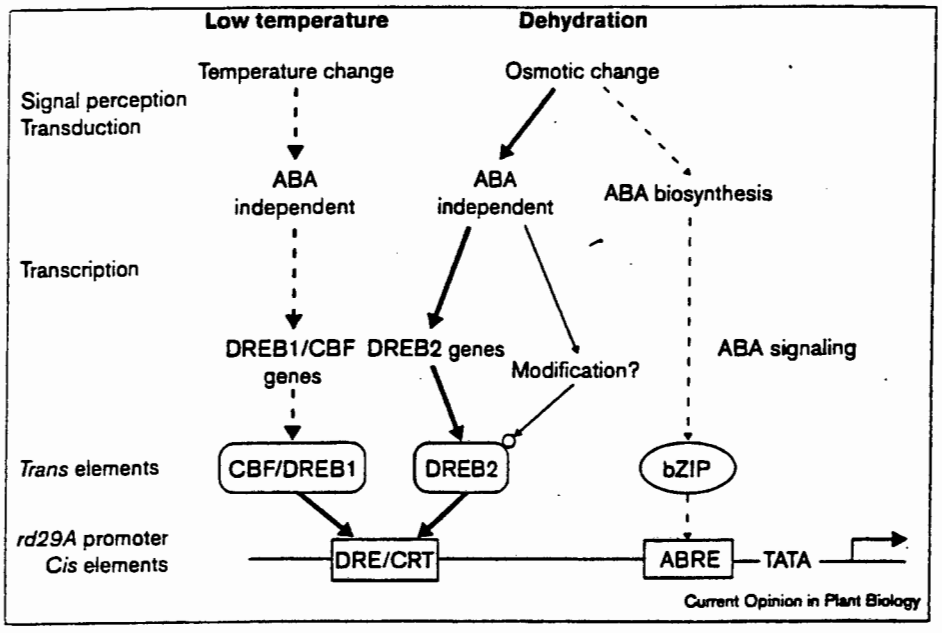

Figure 1.4: A model of the induction of the *rd29A* gene and *cis*- and *trans*-acting elements involved in stress-responsive gene expression. A drought-responsive element (DRE) and an abscisic acid (ABA) responsive element (ABRE) are involved in the ABA-independent and ABA-responsive induction of *rd29A*, respectively. DREB1 and DREB2 separate two different signal transduction pathways in response to cold and drought stresses, respectively (Adapted from Shinozaki *et al.*, 1999 and Shinozaki and Yamaguchi-Shinozaki, 2000).

As can be seen from Figure 1.4, *rd29A* can also be regulated in an ABA-dependent manner. The stresses induce the expression of bZIP, a DNA binding protein, to activate ABRE-mediated expression of the gene (Yamaguchi-Shinozaki and Shinozaki, 1994; Liu *et al.*, 1998; Shinozaki *et al.*, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000).

ABA Response Element

The consensus for the ABRE has been found to be RYACGTGGYR, where R is a nucleotide containing a purine base and Y is a nucleotide containing a pyrimidine base (Shen and Ho, 1995; Bray, 1997; Bonetta and McCourt, 1998). ABREs were first identified in the wheat *Em* gene, which is functional mainly in seed during late embryogenesis (Guiltinan *et al.*, 1990) and in the rice *rab16* gene, which is expressed in maturing seeds as well as in dehydrated vegetative tissues (Mundy *et al.*, 1990).

EmBP-1 encoding a basic leucine zipper (bZIP) protein was identified in wheat. This protein contains a basic DNA-binding domain linked to a leucine zipper motif that is characteristic of transcription factors (Guiltinan *et al.,* 1990). Interestingly, the ABRE core element, ACGT, is also found in other DNA elements including the G box which is involved in the regulation of gene expression by light, auxin, jasmonic acid and salicyclic acid (Menkes *et al.,* 1995). DNA-binding studies have shown that nucleotides around the ACGT core motif are important for determining the specificity of bZIP proteins and subsequent gene activation (Iwaza *et al.,* 1993).

It has also been shown that a single copy of ABRE is not sufficient for ABA-responsive transcription (Skriver *et al.,* 1991) and that at least two copies of ABREs are required for the dehydration responsive expression of *rd29B*, an *Arabidopsis* gene (Uno *et al.,* 2000). Also, even though the ABRE has been shown to be sufficient for ABA-regulated gene expression, in some genes it must be associated with a coupling element. For example, the wheat *HVA22* gene is regulated by an ABA-responsive complex of an ABRE and a coupling element, CE1 (TGCCACCG) (Bray, 1997). Most of the known coupling elements are similar to ABREs and they contain an A/GCGT motif (Hobo *et al.,* 1999). The specific sequence of the coupling element may affect the specificity of ABA-driven gene expression and possibly explain the differences between functional and non-functional ABREs (Ingram and Bartels, 1996).

The yeast one hybrid system was used to isolate 3 basic leucine zipper bZIP-type ABRE binding proteins (AREB1, AREB2, AREB3) from *Arabidopsis* (Uno *et al.*, 2000). Two of these were found to be upreglated by drought, NaCl and ABA. Studies with these AREBs revealed that they function as transcriptional activators of ABA-inducible expression of *rd29B* (Uno *et al.,* 2000), the promoter of which contains an ABRE (Yamaguchi-Shinozaki and Shinozaki, 1994). Furthermore, the ABA-dependent postranscriptional activation of these AREBs, possibly via phosphorylation, is required for maximum activation by ABA (Uno *et al.,* 2000).

Therefore, even though it has been suggested that regions flanking the ACGT core are important for the regulation of genes controlled by AREBs, the coupling elements together with transcriptional activators, add another dimension to the control of gene expression by ABA during water deficit (Bray, 1997).

1.3.4. Gene expression and the function of dehydration-induced proteins

A number of genes have been found to be expressed in response to water deficit stress in various plants. The functions of many of the encoded proteins have been predicted from sequence homology with known proteins (Shinozaki and Yamaguchi-Shinozaki, 1996). These proteins can be classified into two groups, those that take part in the signal transduction pathways (regulatory proteins, Figure 1.5), and those that are end products of these molecular events (functional proteins, Figure 1.5) (Shinozaki and Yamaguchi-Shinozaki, 1997; Shinozaki *et al.*, 1999). Proteins from the first group include factors involved in regulation of signal transduction and gene expression that function in the stress response such as protein kinases, transcription factors and PLC. These have already been dealt with in section 1.3.1.

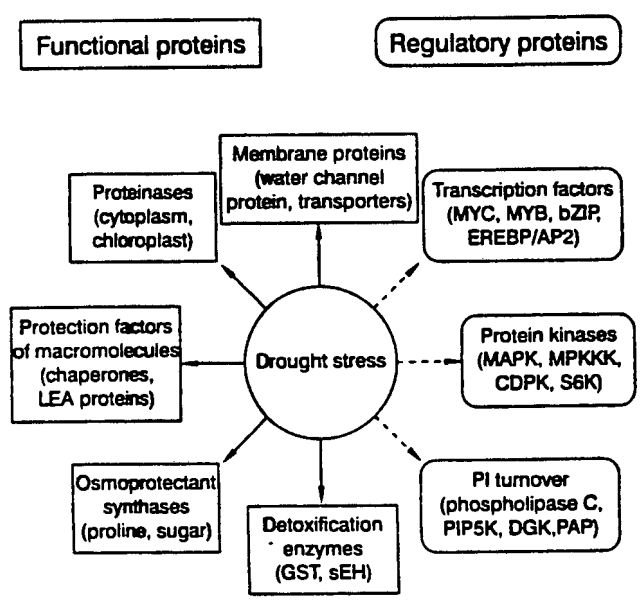

Figure 1.5: Function of water-stress-inducible gene products in stress tolerance and stress response. The gene products are roughly characterised into two groups: functional proteins that are involved in water-stress tolerance and cellular adaptation, and regulatory proteins that may function in gene expression and signal transduction in stress response. MAPK, mitogen-activated kinase; MAPKKK, mitogen-activated protein kinase kinase kinase; S6K, ribosomal S6 protein kinase; CDPK, calcium-dependent protein kinase (Adapted from Shinozaki *et al.*, 1999).

The second group contains proteins that are involved in stress tolerance and includes enzymes responsible for the synthesis of compatible solutes and removal of reactive oxygen species, water channels, ion transporters and protective proteins (Bohnert *et al.*, 1995; Ingram and Bartels, 1996; Bray, 1997; Shinozaki and Yamaguchi-Shinozaki, 1997). The functions of these proteins serve to avoid water deficit, to protect cellular machinery during water deficit as well as to repair damage that may occur during the stress (Bray, 1997).

Mechanisms to avoid water deficit

Both ion and water channels are likely to play an important role in the avoidance of water deficit or osmoregulation during the water deficit stress (Ingram and Bartels, 1996; Bray, 1997). Potassium channels are responsible for the regulation of potassium ion uptake and may also regulate sodium ions (Bray, 1997). Although water can move through the lipid bilayer of a cell passively by osmosis, aquaporins (a family of proteins involved in water transport) have an important role in regulating water transport across the cellular membrane, and have been suggested to be involved in controlling the water content of a cell in response to water deficit (Ingram and Bartels, 1996; Bray, 1997; Shinozaki and Yamaguchi-Shinozaki, 1997).

Another response of plants to water deficit is the synthesis of osmolytes (compatible solutes). These are highly soluble compounds that carry no net charge at physiological pH and are non-toxic at high concentrations. There are three types of osmolytes: betaines and allied compounds, polyols and sugars (e.g. mannitol and trehalose), and amino acids, such as proline. These compounds serve to raise the cytoplasmic osmotic pressure, thereby maintaining cellular turgor and can also stabilise proteins and membranes (Scott, 2000; Hoekstra *et al.*, 2001). Thus, upregulation of enzymes involved in the synthesis of these osmolytes permits osmotic adjustment or the net accumulation of solutes. This results in a decreased osmotic potential which may in turn maintain a favourable water potential gradient thereby preventing water loss. Also if water is lost, these compounds are able to replace water molecules and stabilise proteins, membranes, proteins and maintain cellular turgor (Ingram and Bartels, 1996; Bray, 1997; McNeil *et al.,* 1999; Scott, 2000; Hoekstra *et al.,* 2001).

Mechanisms to protect the cellular machinery

Proteins that may protect macromolecules and membranes have also been found to be induced during dehydration. These include proteins such as LEA (Late Embryogenesis Abundant) proteins, osmotin, antifreeze, chaperones and mRNA binding proteins (Shinozaki and Yamaguchi-Shinozaki, 1997). LEA proteins were first identified in seeds during the final desiccation stage of development and have also been found in vegetative tissue subjected to desiccation (Ingram and Bartel, 1996; Bray, 1997). LEA proteins are extremely hydrophilic and have, therefore, been predicted to play various roles during dehydration including; sequestration of ions, binding of water and they may operate as molecular chaperons (Bray, 1997). Molecular chaperones protect proteins during conditions of stress

that result in protein unfolding. As water leaves the cell, proteins become damaged and may unfold and interact with other cellular components. Molecular chaperones act to prevent aggregation of unfolded proteins and promote renaturation of aggregated proteins. They bind to and stabilise denatured proteins in attempt to reduce any further damage (Gething and Sambrook, 1992; Hendrick and Hartl, 1993; Morimoto *et al.,* 1994; Parsell and Linquist, 1994; Boston *et al.,* 1996; Hartl, 1996; Johnson and Craig, 1997; Netzer and Hartl, 1998).

Mechanisms to repair damage

Proteins involved in limitation of damage as well as the removal of toxic compounds are induced during water deficit (Ingram and Bartels, 1996; Bray, 1997; Shinozaki and Yamaguchi-Shinozaki, 1997). Oxygenic metabolism occurring during water deficit can result in the production of free radicals which can have deleterious effects on membranes, proteins and DNA (McAinsh *et al.,* 1996), it is important therefore to ensure their rapid and efficient removal. Enzymes and their activity concerned with the removal of these toxic intermediates, such as glutathione reductase, catalase, ascorbate peroxidase and superoxide dismutase, increase in response to drought stress and are probably very important in tolerance (Smirnoff, 1993; Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1997; Farrant, 2000). Recently, it has been proposed that enzymes responsible for the synthesis of compatible solutes as well as compatible solutes themselves, may act as detoxification enzymes or as radical scavengers, respectively (Oberschall *et al.,* 2000; Akashi *et al.,* 2001).

During desiccation stress, protein residues may be chemically modified (e.g. deamination or oxidation) and these proteins must be degraded or repaired to ensure the survival of the plant (Ingram and Bartels, 1996). Proteins such as thiol protease and ubiquitin are involved in targeting and degradation of damaged or denatured proteins (Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1997). Again there is a requirement for molecular chaperones. They are involved in the repair and refolding of proteins that have been damaged during the stress as well as targeting irreparably damaged proteins for degradation (Gething and Sambrook, 1992; Hendrick and Hartl, 1993; Morimoto *et al.,* 1994; Parsell and Linquist, 1994; Hartl, 1996; Johnson and Craig, 1997; Netzer and Hartl, 1998). There is increasing evidence for the importance of molecular chaperones, such as the small heat shock proteins, Hsp70 and Hsp90 (the subject of this thesis) in the recovery of proteins during environmental stresses, such as dehydration and high temperature in plants (Boston *et al.,* 1996; Miernyk, 1999).

1.4. Molecular chaperones

1.4.1. Hsp90

Hsp90 belongs to a group of highly conserved proteins called Heat Shock Proteins. As this name suggests, these proteins were originally discovered to be induced under conditions of heat stress, but subsequent to this they were also found to have essential functions during normal cellular processes (Craig *et al.,* 1993; Georgepoulus and Welch, 1993; Hendrick and Hartl, 1993; Parsell and Lindquist, 1993; Morimoto *et al.,* 1994; Boston *et al.,* 1996; Fryman and Hohfeld, 1997; Netzer and Hartl, 1998). The Hsp90 family of proteins are molecular chaperones which are structurally diverse and have a common function that involves the binding of non-native proteins and facilitation of the correct folding of the protein. Chaperones bind to the hydrophobic regions of newly synthesised or denatured proteins to prevent the incorrect interaction of the unfolded polypeptide with other cellular components (Ellis and van der Vies, 1991; Hendrick and Hartl, 1993; Parsell and Lindquist, 1993; Morimoto *et al.,* 1994; Boston *et al.,* 1996; Netzer and Hartl, 1998; Miernyk, 1999). These proteins are distributed throughout the cell (Parsell and Linquist, 1994; Boston *et al.,* 1996; Johnson and Craig, 1997), with homologues in each compartment including the cytosol, nucleus and specialised organelles such as the endoplasmic reticulum and chloroplasts (Morimoto *et al.,* 1994; Boston *et al.,* 1996) and are therefore able to act at many stages of a protein's life (Johnson and Craig, 1997). They are able to function independently or as part of a complex, referred to as chaperone machines (Pratt, 1993; Johnson and Craig, 1997; Pratt and Toft, 1997). The affinity of the chaperone for polypeptide substrates is modulated by the partner proteins in the chaperone machine and conformational changes induced by ATP binding and hydrolysis (Prodromou *et al.,* 1999, 2000; Weikl *et al.,* 2000; Hartl and Young, 2000). Partner proteins have been postulated to modulate chaperone function in a number of ways, including direct interaction with chaperone-bound substrates, direct interaction with the chaperone to modulate its interaction with the protein substrate, or modulation of the rate of ATP hydrolysis or nucleotide exchange (Hutchinson, 1995; Thulasiraman and Matts, 1996; Hartl and Young, 2000).

Different molecular chaperones have different functions and these include facilitating folding of nascent proteins, promoting folding of proteins to their final conformation, holding polypeptides in an unstructured form competent for transport across membranes, maintaining proteins in specific conformations required for their proper functioning, and preventing aggregation of unfolded proteins and promoting renaturation of aggregated proteins (Gething and Sambrook, 1992; Hendrick and Hartl, 1993; Morimoto *et al.,* 1994;

Parsell and Linquist, 1994; Boston *et al.,* 1996; Hartl, 1996; Johnson and Craig, 1997; Netzer and Hartl, 1998). The latter two functions are particularly important for cells experiencing stresses such as high temperature or desiccation (Parsell and Lindquist, 1994; Csermely *et al.,* 1998; Netzer and Hartl, 1998). Some examples of molecular chaperones and their specific functions are shown in Table 1.1.

During normal cellular processes protein stability depends on proper folding. Newly synthesised proteins need help while folding, as do proteins that must move to different parts of the cell and proteins that must be held in specific conformations in order to perform their functions properly (Morimoto *et al.,* 1994; Hartl, 1996; Boston *et al.,* 1996; Netzer and Hartl, 1998). These processes involve the transient unfolding of the polypeptide and therefore exposure of interactive protein surfaces to the intracellular environment. Unfolded polypeptides expose hydrophobic amino acid side chains which are sticky and therefore act as "hot spots" for protein aggregation. (Hendrick and Hartl, 1993; Hartl, 1996; Johnson and Craig, 1997; Netzer and Hartl, 1998). *In vivo* the problem of protein aggregation is acute primarily because of the high intracellular protein concentration (150 mg/ml) and the presence of these hydrophobic interactions (Morimoto *et al.,* 1994; Hartl, 1996; Miernyk, 1999). This problem is made even more acute during conditions of stress which result in protein denaturation (e.g. heat, dehydration) and the accumulation of exposed hydrophobic domains (Hendrick and Hartl, 1993; Morimoto *et al.,* 1994, Hartl., 1996; Miernyk, 1999). Molecular chaperones recognise and bind these exposed hydrophobic residues protecting the protein as it folds and thereby preventing aggregation (Ellis and van der Vies., 1991, Gething and Sambrook., 1992; Parsell and Lindquist, 1993; Morimoto *et al.,* 1994; Hartl, 1996; Netzer and Hartl., 1998; Miernyk, 1999). The chaperones do not contain any steric information (Parsell and Lindquist, 1993; Hartl, 1996) as the primary amino acid sequence of a polypeptide contains all the information necessary for a protein to fold (Anfisen, 1973). Instead they prevent incorrect interactions within and between non-native polypeptides, thus typically preventing aggregation and thereby directing the polypeptide into productive folding, transport or degradation pathways. They may also facilitate the dissociation of aggregates that may have formed (Boston *et al.,* 1996; Johnson and Craig, 1997; Netzer and Hartl, 1998).

Hsp90 is one of the most abundant heat shock proteins found in eukaryotic cells. Its levels amount to 1-2% of the total cellular protein under normal growth conditions and can be further induced by heat shock or other environmental stresses (Gething and Sambrook, 1992; Parsell and Lindquist, 1993; 1994; Bohen and Yamamoto, 1994; Csermely *et al.,*

1998; Buchner, 1999). Members of the Hsp90 gene family are well conserved from bacteria through to humans with an overall amino acid identity of 40% (Gething and Sambrook, 1992; Parsell and Lindquist, 1993; Gupta, 1995). The majority of cellular Hsp90 is found in the cytoplasm, but distinct organellar forms have been found in the endoplasmic reticulum (called Grp94 or endoplasmin), plastids and mitochondria of eukaryotic cells (Boston *et al.*, 1996; Mogelsvang and Simpson, 1998). Members of the Hsp90 family range in size from 82 to 96 kDa but despite this variation in size and the high levels of expression under non-stress conditions, the term Hsp90 has remained (Gething and Sambrook, 1992; Miernyk, 1999).

Table 1.1. Major classes of foldases and molecular chaperones in eukaryotes (Adapted from Boston *et al.*, 1996).

Protein class[a]	Intracellular location	Prokaryotic homologue
Chaperones		
Clp proteins		
Hsp100 (ClpB)	Cytoplasm, mitochondrion	ClpB
ClpA/C	Chloroplast	ClpA/C
Hsp90		HtpG
Hsp80/90	Cytoplasm	
Grp94	ER	
Hsp70		DnaK
Hsp/Hsc70	Cytoplasm,nucleus,chloroplast, mtochondrion	
BiP/Grp78	ER	
Chaperonins		GroEL
Hsp60/Cpn60	Chloroplast, mitochondrion	
TRIC/TCP-1	Cytoplasm	TF55
Calnexin	ER	
Nucleoplasmin	Nucleus	
Small HSPs	Cytoplasm, mitochondrion, chloroplast, ER[b]	IBPA/B
Co-chaperones[c]		
DnaJ/Hsp40(Hsp70)	Cytoplasm, mitochondrion, ER	DnaJ
GrpE(Hsp70)	Mitochondrion	GrpE
Cpn10(Cpn60)	Mitochondrion, chloroplast	GroES
Foldases		
Protein disulphide isomerase	ER	DsbA
Peptidly prolyly isomerase (or immunophlins)		
Cyclophilin	Cytoplasm, ER, mitochondrion	PPIa,b
FK506 binding protein	Cytoplasm, ER	

[a] Only the major members of each class are listed, in many cases multiple alternative names have appeared in the literature.
[b] There is no specific nomenclature for the small Hsps found in different cellular compartments; they are referred to as small Hsps with a molecular weight value.
[c] The major chaperone with which each protein interacts appears in parantheses.

1.5. Cellular functions of Hsp90 and Grp94

The 90-kDa heat shock protein family members are the least understood of all the major heat shock proteins in terms of their cellular function (Buchner, 1999). Studies have concentrated on the function of the cytosolic member of this family, Hsp90, and as a consequence very little is known about Grp94, the ER homologue. This protein is one of the most abundant chaperones within the ER and is believed to be responsible for the correct folding and assembly of proteins passing through the ER (Koch *et al.,* 1986; Melnick *et al.,* 1992, 1994; Muresan and Arvan, 1997; Chavany *et al.,* 1996; Linnik and Herscovitz, 1998; Csermely *et al.,* 1998;).

Hsp90 is able to prevent the aggregation of unfolded proteins and cooperates with the hsp70/hsp40 chaperone system in the ATP-dependent refolding of unfolded model proteins *in vitro* (Weich *et al.,* 1992; Jakob *et al.,* 1995; Freeman and Morimoto *et al.,* 1996; Young *et al.,* 1997). However, *in vivo* studies indicate a more restricted role for Hsp90 in signal transduction via interactions with certain signaling molecules, including steroid hormone receptors and several protein kinases (Picard *et al.,* 1990; Pratt, 1993; Xu and Lindquist, 1993; Alique *et al.,* 1994; Bohen and Yamamoto, 1994; Bohen *et al.,* 1995; Hartson *et al.,* 1996; Dittmar *et al.,* 1997; Fryman and Hohfeld, 1997; Johnson and Craig, 1997; Pratt and Toft, 1997; Buchner, 1999). Specific Hsp90 client proteins in mammalian cells include tyrosine kinases such as $pp60^{v\text{-}src}$ (Xu and Lindquist, 1993; Mimnaugh, 1995; Hunter and Poon, 1997) and Sevenless (Cutforth and Rubin, 1994); serine/threonine kinases such as Wee1 (Alique *et al.,* 1994), c-Raf (Stancato *et al.,* 1993), and Cdk4 (Dai *et al.,* 1996); helix-loop-helix transcription factors (Wilhelmson *et al.,* 1990); tumor suppressers such as Rb (Chen *et al.,* 1996), cytoplasmic steroid hormone receptors (Pratt, 1993; Fang *et al.,* 1996) and tubulin (Sanchez *et al.,* 1988), amongst others. In addition, Hsp90 has been shown to participate in the renaturation of a diverse range of proteins that have unfolded under conditions of cellular stress (Schneider *et al.,* 1996; Nathan *et al.,* 1997, Pratt and Toft, 1997). In performing these functions Hsp90 interacts with a range of accessory proteins including p50/CDC37, Hsp70/DnaK, Ydj/DnaJ, p60/Hop/Sti1, p23 and various immunophilins (FKBP52, Cyp-40), several of which have been identified as molecular chaperones themselves (Czar *et al.,* 1994; Kimura *et al.,* 1995; Bose *et al.,* 1996; Duina *et al.,* 1996; Freeman and Morimoto, 1996). Much of what is known about the structure and function of the chaperone complex has been obtained from studies on the Hsp90s role in steroid hormone receptor activation and is discussed at length in section 1.5.1.

Grp94 is the most abundant chaperone in the ER (Koch *et al.,* 1986), and it has been proposed to perform chaperone functions in the folding, assembly and secretion of nascent polypeptides (Schaiff *et al.,* 1992; Melnick *et al.,* 1992, 1994; Chavanny *et al.,* 1996; Vitale and Denecke, 1999). Grp94 expression is up-regulated by stress conditions that promote protein misfolding or unfolding in the ER (Kozutsumi *et al.,* 1988; Lenny and Green, 1991; Lee, 1992; Bush *et al.,* 1997). It is also involved in the loading of proteosome-generated antigenic peptides onto nascent major histocompatability complex class I molecules (Li and Srivastava, 1993). Thus in addition to its role in protein folding and assembly Grp94 appears to function in an as yet unclear manner as part of the MHC class I antigen processing and presentation pathways of mammalian cells (Srivastava, 1986; Li and Srivastava, 1993; Suto and Srivastava, 1995; Nieland *et al.,* 1996).

1.5.1. Hsp90 in signalling

Hsp90 in the steroid response

As mentioned above, one group of the major cellular targets of Hsp90 are signal transducers. These are unstable proteins that, through repeated cycles of binding and release by Hsp90, are held ready for activation until they are stabilised by conformational changes associated with signal transduction (Nathan and Lindquist, 1995). The most well studied example of Hsp90 function is that of steroid hormone activation and much of what is known about Hsp90 function has been obtained from studies of this process (Figure 1.6).

Hsp90 together with its partner proteins have been implicated in both the initial folding of steroid hormone receptors and in the modulation of their DNA binding and transcriptional regulatory activities (see Figure 1.6; Picard *et al.,* 1990; Bohen and Yamamoto, 1993; Nathan and Lindquist, 1995). Hsp90 interacts with hydrophobic domains of the newly synthesised aporeceptors to prevent their internal collapse or intermolecular aggregation and thereby maintaining a conformation that can readily bind ligand (Bohen and Yamamoto, 1993; Pratt, 1993; Smith, 1993; Bohen and Yamamoto, 1994; Fang *et al.,* 1996). Only Hsp90-bound receptors are capable of binding steroid hormone (Jakob and Buchner, 1994) and hormone binding promotes Hsp90 dissociation from the complex thus allowing the receptor to bind to specific DNA sequences and activate transcription (Smith and Toft, 1993; Pratt, 1997). Hsp90 therefore appears to have a role in maintaining the receptors in a conformation competent to bind hormone. This property of Hsp90 depends on the presence of Hsp90 co-chaperones and partner proteins (Table 1.2; Johnson and Craig, 1997; Pratt

and Toft, 1997; Buchner, 1999). Three distinct complexes are observed during the maturation of receptors from the inactive apo-receptor to the active hormone-bound transcription factor (see Figure 1.6). An early complex consisting of Hsp70 and Hsp40 is followed by an intermediate complex with Hsp90, Hsp70 and Hop, finally completed by the mature complex containing Hsp90, p23, and one of the large immunophilins (Smith *et al.*, 1993; Nair *et al.*, 1996).

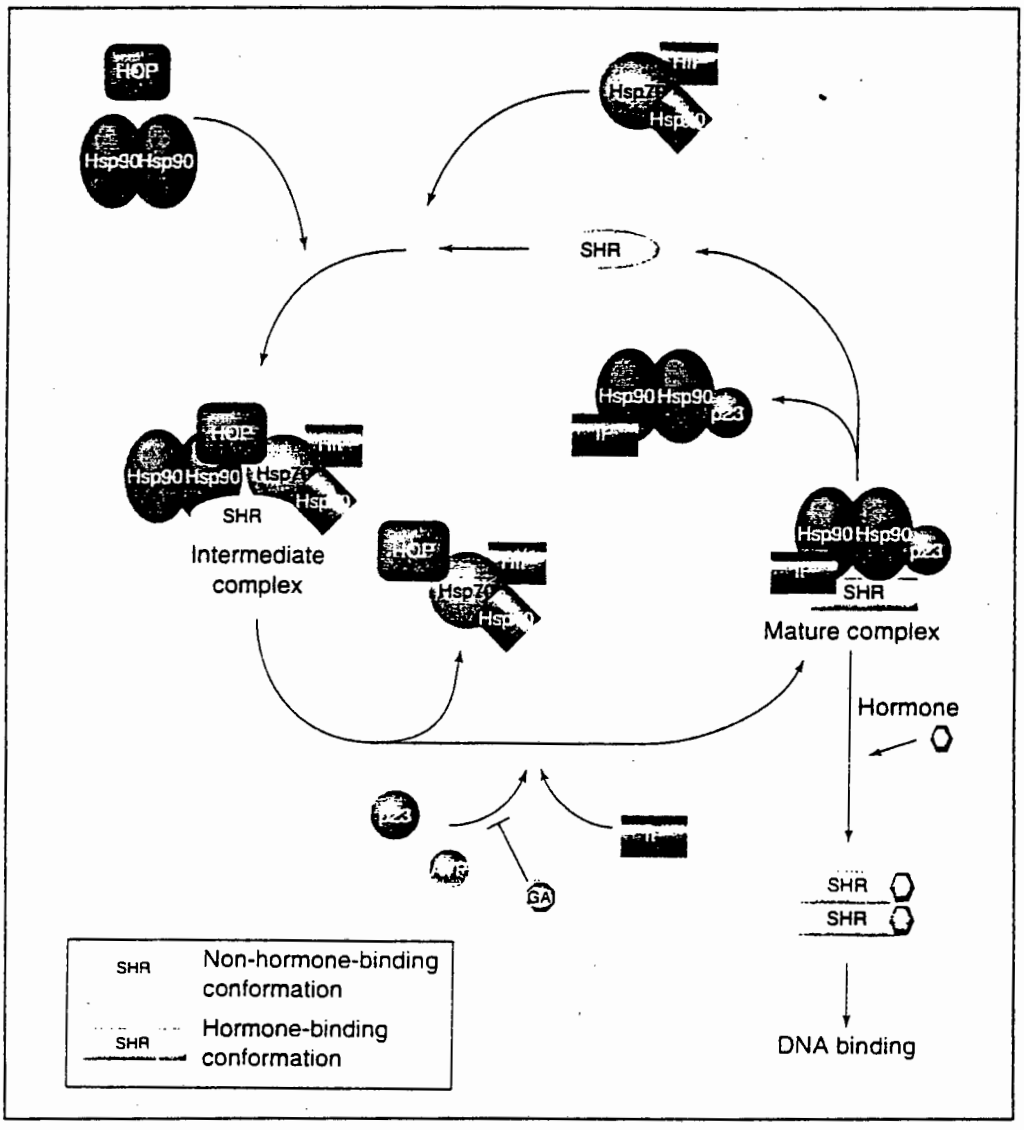

Figure 1.6: Model of glucocorticoid receptor activation by Hsp90. The Hsp90/Hsp70-based chaperone machinery converts the glucocorticoid receptor (GR) ligand binding domain from a folded conformation (in which the hydrophobic binding cleft is closed and not accessible to hormone) to a partially unfolded conformation (in which the cleft is opened and accessible to steroid). Hsp70 binds Hip (a cofactor) and binds to the GR in an ATP-dependent and a Hsp40-dependent step to form a 'primed' GR-Hsp70 complex that can then bind to Hsp90. The scaffold protein, Hop, brings the hsp70 and Hsp90 complexes together. One molecule of Hop is bound per Hsp90 dimer. Hsp90 facilitates the opening of the steroid binding cleft through cycles of binding and release. The Hsp70 components dissociate and at the same time, p23 and one of the large immunophilins (IP) enter the complex. p23 is believed to stabilise the complex and enhance substrate release. The steroid hormone receptor is released from the complex and it can bind hormone, dimerise and bind DNA. (Adapted from Buchner, 1999 and Pratt *et al.*, 2001).

Table 1.2. Hsp90-partner proteins (Buchner, 1999).

General name	Name in higher eukaryotes	Function
Hsp70	Hsp70	Chaperone
p23	P23	Chaperone
HiP (Hsp70 interacting protein)	Hip, p48	Hsp70 cofactor
Hsp40	Hsp40, Hdj1	Hsp70 cofactor
Hop (hsp organising protein)	Hop, p60	Assembly factor for Hsp90 and Hsp70
Cdc37	P50	Kinase-specific cofactor
FKBP51	FKBP51, p54, FKBP54	Prolylyisomerase, chaperone
FKBP52	FKBP52, p59, p56, HBI, Hsp56, FKBP73, FKBP77	Prolylyisomerase, chaperone
Cyp40	Cyp40	Prolylyisomerase, chaperone
PP5	PP5	Protein-serine phosphatase, prolyly isomerase?

Hsp90 and protein kinases

Hsp90 is required for the correct folding and therefore the activity of many of kinases as mentioned above. Viral $pp60^{src}$ kinase (v-SRC) is a well-characterised substrate of Hsp90. The active form of this kinase is attached to the plasma membrane. When v-Src is bound to Hsp90 it lacks kinase activity as it is in a hypophosphorylated state. As Hsp90 and v-Src dissociate they both undergo multiple phosphorylations, and v-Src gains kinase activity and associates with the membrane (Mimnaugh *et al.,* 1995; Hunter and Poon, 1997). Hsp90 is complexed with the immature cytosolic form of V-Src, but not with the native membrane bound form, which indicates an important role for Hsp90 during the maturation of the kinase (Xu and Lindquist, 1993). A kinase specific 50-kDa protein (p50) acts together with Hsp90 during this process (Hunter and Poon, 1997) and Hop, a protein involved in the formation of steroid receptor-folding chaperone complexes, is also involved in this complex (Owens-Grillo *et al.,* 1996).

It is of interest that the Hsp90 homologue found in the ER, Grp94, also seems to form complexes with kinases (Csermely *et al.,* 1998). Recent studies showed that Grp94 forms a complex with p185-erbB2, which is a receptor-like kinase that is overexpressed in many forms of cancer, including breast and ovarian cancer (Chavany *et al.,* 1996). It is also known that Grp94 is associated with protein kinase CK-II (Cala and Jones, 1994; Csermely *et al.,* 1995; Trujillo *et al.,* 1997).

1.5.2. Hsp90-A folding helper protein *in vitro*

In order to perform their functions, molecular chaperones must recognise and transiently bind to misfolded or unfolding protein intermediates that are susceptible to aggregation, thereby suppressing non-specific side reactions and increasing the yield of properly folded

proteins (Ellis and van der Vies, 1991; Hendrick and Hartl, 1993; Parsell and Lindquist, 1993; Morimoto *et al.,* 1994; Boston *et al.,* 1996; Netzer and Hartl, 1998; Miernyk, 1999). *In vitro* studies have been performed using model substrate proteins that have been either chemically or heat denatured to assess the effect of Hsp90 on proteins that are susceptible to aggregation.

These *in vitro* studies have shown that Hsp90 is able to suppress the aggregation of both chemically and thermally denatured proteins. In the early 1990's Wiech *et al.* (1992) used light scattering experiments to show that Hsp90 suppressed the aggregation of guanidine-denatured citrate synthase, thereby resulting in an increased yield of correctly folded functional protein. Another protein with different secondary structure composition and different folding characteristics, namely the Fab fragment of a monoclonal antibody, was also tested and Hsp90 was again shown to increase the yield of this protein in the native state (Wiech *et al.,* 1992). This was further confirmed by studies with protein kinase CK-II at low ionic strength (Miyata and Yahara, 1992, 1995). However, it has not been shown to be able to promote the disaggregation of severely denatured protein kinase CK-II aggregates (Miyata and Yahara, 1995). Further studies with heat-denatured luciferase (Schumacher *et al.,* 1996), heat-denatured citrate synthase (Jakob *et al.,* 1995) and gaunidium-denatured β-galactosidase (Freeman and Morimoto, 1996) indicated that although Hsp90 suppressed aggregation of the denatured proteins, it was not able to refold these proteins alone. Analysis of the kinetics of citrate synthase unfolding has revealed that Hsp90 suppresses aggregation by binding to early unfolding intermediates (Jakob *et al.,* 1995). Pharmacological and immunological approaches were used to confirm the role of Hsp90 and identify other components involved in the renaturation of thermally denatured luciferase (Thularisman and Matts, 1996). Geldanamycin, which blocks Hsp90 function, was shown to inhibit the renaturation of denatured luciferase in reticulocyte lysates. When heat-denatured luciferase was added to reticulocyte lysate it was shown to coimmunoprecipitate with Hsp90, Hsp70, p60, Hip and p23, thus suggesting a role for this entire complex of proteins in the folding process (Schumacher *et al.,* 1996; Thularismann and Matts, 1996). The results above indicate that although Hsp90 does not possess refolding activity, it interacts with non-native proteins with a high degree of secondary structure, preventing their aggregation and maintaining them in folding competent conformations that can complete folding upon the addition of other chaperones (Jakob *et al.,* 1995; Freeman and Morimoto, 1996; Yonehara *et al.,* 1996). Some *in vitro* observations show that, indeed, the chaperone activity of Hsp90 is activated at higher temperatures, corresponding to the usual range of cellular heat shock (Jakob *et al.,* 1995a; Yonehara *et al.,* 1996). *In vitro* Hsp90 was also shown to retain

partially denatured proteins in a folding-competent state (Freeman and Morimoto, 1996), which may be an important mechanism of its *in vivo* rescue function after cellular stress.

1.5.3. Hsp90 and stress response

As yet it remains to be determined whether Hsp90 functions to facilitate the refolding of denatured proteins in response to stress. As stated earlier, Hsp90 is a very abundant protein under normal conditions, but its levels increase even more after cellular stress, such as heat shock (Gething and Sambrook, 1992; Parsell and Lindquist, 1993; Buchner, 1999). Despite this important fact, very few *in vivo* experiments have been conducted to study the function of Hsp90 during stress in the cell. Schneider *et al.* (1996) were able to show that the Hsp90 inhibitor, geldanamycin inhibits the refolding of firefly luciferase in vertebrate cells after heat shock. This is in agreement with results obtained from *in vitro* studies with heat denatured luciferase in rabbit reticulocyte lysate (Thularisman and Matts, 1996). It has been demonstrated that Hsp90 deletion mutants of *E. coli* exhibit slower growth at higher temperatures than wild type (Bardwell and Craig, 1988). It was also shown that Hsp90 is essential for survival of yeast at all temperatures (Borkovich *et al.,* 1989) and that decreased intracellular Hsp90 concentration leads to an increased mortality of mammalian cells at elevated temperatures (Bansal *et al.,* 1991).

The *in vitro* studies together with these *in vivo* observations certainly hint at a role for Hsp90 in the refolding of proteins damaged due to stress. The exact role as yet is still unclear. Recent data of Nathan *et al.* (1997) suggest that Hsp90 generally does not protect proteins from thermal inactivation, but enhances the rate at which a heat-damaged protein is reactivated. This is again in agreement with the *in vitro* studies mentioned above. Hsp90 was shown to bind to unfolding proteins and hold them in a state that is competent to fold. Folding is then completed once the other chaperones of this folding complex are present. This could be an explanation for the vast amounts of the protein being present; it is able to bind the unfolding proteins very quickly upon stress and hold them out of harms way until they are refolded again.

1.5.4. Hsp90, a general chaperone

It remains to be determined whether Hsp90 has a role as a general chaperone, assisting the folding of a range of proteins *in vivo*. The abundance of Hsp90 in the cell, as well as its apparent promiscuity *in vitro*, suggests a role as a general chaperone, however its only conclusively determined function *in vivo* is the conformational regulation of receptors and tyrosine kinases (Csermely *et al.,* 1998). Assisted folding is a vital function that requires a

large amount of the chaperone (Csermely *et al.,* 1998). Recently evidence has suggested Hsp90-independence of *de novo* folding of most proteins. Nathan *et al.* (1997) set out to determine possible functions of Hsp90 *in vivo* using a unique temperature sensitive mutant of *Saccharomyces cerevisiae.* First the role of Hsp90 in *de novo* protein folding was investigated. A known *in vivo* substrate of Hsp90, the oncogenic tyrosine kinase p60-v-src, as well as *in vitro* substrates β-galactosidase and firefly luciferase, were expressed in Hsp90-deficient yeast cells and their activity was monitored. Bacterial luciferase protein fusions were also tested and in all cases it was found that Hsp90 did not have a role in *de novo* folding. The general role of Hsp90 in folding of yeast proteins was then examined by comparing the aggregation of newly synthesised proteins in wild type and Hsp90 deficient yeast cells. The results suggested that Hsp90 is not involved in the folding of the majority of yeast proteins. The role of Hsp90 in preventing the thermal inactivation of proteins *in vivo* was also determined. Substrate proteins were expressed in Hsp90-deficient yeast cells and subsequently exposed to various temperatures to monitor enzyme expression. Results showed that Hsp90 is unlikely to play a role in protecting most proteins from thermal inactivation. The recovery of heat damaged firefly luciferase was compared in the presence and absence of functional Hsp90 and it was shown that Hsp90 helped recover this heat damaged protein (Nathan *et al.,* 1997).

1.5.5. Other functions of Hsp90

Other functions have been proposed for Hsp90 and a brief overview is presented here. Hsp90 has been found to be able to crosslink actin *in vitro* (Koyasu *et al.,* 1986; Nishida *et al.,* 1986; Kellermayer and Csermely , 1995) and it has been suggested that *in vivo* Hsp90 forms stable complexes with actin filaments after severe stress, when there is a significant drop in cellular ATP levels (Csermely *et al.,* 1998). It has also been found that Hsp90 binds tubulin (Sanchez *et al.,* 1988; Redmond *et al.,* 1989; Fostinis *et al.,* 1992) and it is thought that it may have a role in protecting microtubules after heat shock (Williams and Nelsen, 1997). Hsp90 has also reportedly been found to be associated with non-microtubular and non-microfilamental structures of the cytoplasm, which are similar to intermediate filaments (Fostinis *et al.,* 1992). These results suggest a role for Hsp90 in the organisation of the cytoplasm and a possible protective role after cellular stress to preserve the structural integrity of the cell (Csermely *et al.,* 1998).

The cell nucleus is known to harbour 5-10% of the cellular hps90 and even more translocates to the nucleus after heat shock (Arrigo *et al.,* 1980; Collier and Schlessinger, 1986). It is thought that Hsp90 may be transported to the nucleus by other components of the Hsp90 complex, such as FKBP52, steroid hormone receptors or certain protein kinases. Hps90 does, however, possess a bipartite nuclear localisation sequence, located in the middle highly charged region of Hsp90 (Nardai *et al.,* 1996), but this may be hidden in interior of the Hsp90 dimers. It has been proposed that Hsp90 is involved in the transport of a group of proteins characterised by certain nuclear hormone receptors and protein kinases, to the cell nucleus (Csermely *et al.,* 1998).

Hsp90 binds to RNA and DNA with fairly low affinity (Szanto *et al.,* 1996) and it has been found in nuclear structures that are actively involved in RNA synthesis and processing (Carbajal *et al.,* 1990; Vazquez-Nin *et al.,* 1992; Biggiogera *et al.,* 1996). The tertiary structure of the Hsp90 N-domain shows significant homology with DNA topoisomerases and DNA gyrases and purified Hsp90 displays topiosomerase/nuclease-like activity, indicating a possible interaction of Hsp90 with DNA (Szanto *et al.,* 1996). Studies with *Drosophila melongaster*, *Chironomus thummi* and *Chironomus tentans* have implicated Hsp90 in DNA rearrangement after heat shock, as well as during embryonic development (Morcillo *et al.,*1993).

Hsp90 has also been suggested to have a role in the modulation of DNA-protein interactions. This idea has arisen from the observation that Hsp90 readily binds histone molecules (Csermely *et al.,* 1997), and both histones H1 and nucleosome core histones display a tighter binding to DNA that is resistant to high salt conditions in the presence of Hsp90 (Csermely *et al.,* 1994). Csermely *et al.* (1994) were also able to show that Hsp90 promotes the assembly of histones and DNA at normal salt concentrations.

Hsp90 also interacts with other DNA-binding proteins, such as transcription factors (Nair *et al.,* 1996; Pratt, 1997). Although the main function of Hsp90 interaction with transcription factors is to aid their maturation, there are observations that suggest a role for Hsp90 in modulating the transcription factors interactions with DNA. A transient low affinity association of Hsp90 with the transcription factor results in enhanced DNA binding (Shaknovich *et al.,* 1992; Shue and Kohtz, 1994). In contrast, it has been shown as with the steroid hormone receptors that a stable complex with Hsp90 in the absence of hormone prevents the receptor from being able to bind to DNA (Csermely *et al.,* 1998).

Thus, although Hsp90 is one of the most abundant chaperones in the cell, it would appear that its confirmed *in vivo* functions are fairly restricted. The results above indicate that Hsp90 is not required for *de novo* folding of most proteins; its main function seems to be the temporary stabilisation of certain unstable molecules (such as steroid hormone receptors and cell signalling kinases) thereby stopping their collapse or aggregation (Nathan *et al.,* 1997).

In vitro studies present Hsp90 as a promiscuous chaperone, interacting with a broad range of folding and unfolding intermediates, whereas as *in vivo* Hsp90 has been found associated only with a small defined set of substrate proteins (Picard *et al.,* 1990; Bohen and Yamamoto, 1993; Xu and Lindquist, 1993; Nathan and Lindquist, 1995). These results appear to be rather conflicting, but, if considered more carefully, it can be seen that there are similarities in both situations. In both cases Hsp90 interacts transiently, binding and releasing, non-native proteins that have a high degree of secondary and tertiary structure. This has been clearly demonstrated for thermally unfolding CS and is well established for steroid hormone receptor and protein kinases. This would suggest that it is probably the native-like structure that characterises all Hsp90 substrates and provides a link between the *in vivo* and *in vitro* substrate specificities of Hsp90.

There is still much speculation concerning the exact role of Hsp90 *in vivo*. Its absence is lethal to yeast and *Drosophila* (Borkovich *et al.,* 1989; van der Straten *et al.,* 1997), and in *Drosophila*, heterozygous mutants display developmental abnormalities (van der Straten *et al.,* 1997; Rutherford and Lindquist, 1998). It is therefore obviously important and has an important role in cell signalling and cell cycle control. But one question that cannot be answered as yet, is why is there so much of the protein? None of the postulated functions require a 1000-fold excess of the protein and would lead one to think that there is more to this protein than is known (Csermely *et al.,* 1998).

1.5.6. Grp94, an ER resident

Grp94 (where Grp is named for glucose-regulated protein) or endoplasmin is a member of the Hsp90 family that resides in the lumen of the endoplasmic reticulum (Koch *et al.,* 1986; Mazarella and Green, 1987; Li and Srivastava, 1993). Grp94 and BiP/Grp78 (Hsp70 homologue) are two of the better characterised ER chaperones (Mazzarella and Green, 1987; Haas and Wabl, 1993). These are abundant proteins in the normal ER but are further induced under stress conditions that result in the accumulation of unfolded proteins in the ER (Kozutsumi *et al.,* 1988; Lenny and Green, 1991; Lee, 1992; Bush *et al.,* 1997; Wearsch

et al., 1998). Calreticulin, calnexin, the protein disulfide isomerase Erp72 and the collagen-specific chaperone Hsp47 are other chaperones that have been found to be localised to the ER (Ferreira *et al.,* 1994, 1996; Tatu and Helenius, 1997; Kuznetsov *et al.,* 1997). An amino terminal signal sequence targets the protein to the ER and the KDEL amino acid motif is responsible for its retention in the ER lumen (Sorger and Pelham, 1987).

The ER is the site where initial folding and processing of newly synthesised secretory proteins occurs and normal function of the ER in protein synthesis, maturation and transport requires the coordinated action of a large family of intrinsic proteins (Gething and Sambrook, 1992; Vitale and Denecke, 1999). The ER chaperones are constitutively expressed in all cells, where they have a major role in monitoring and assisting the maturation of normal proteins (Gething and Sambrook, 1992; Hendrick and Hartl, 1993; Vitale and Denecke, 1999). Most eukaryotic proteins are synthesised in the cytosol and those destined to reside in different compartments of the cell cross ER membrane to the lumen where they are correctly folded and assembled by molecular chaperones before being translocated to their final destination (Vitale and Denecke, 1999). The ER is thus a unique compartment within the eukaryotic cell as it ensures the correct folding and assembly of its own resident proteins and also of proteins destined to other locations within the cell (Vitale and Denecke, 1999).

The discovery of the ER molecular chaperone machinery has led researchers to realise the importance of the ER in safeguarding the correct folding and assembly of proteins within the cell. The ER acts as a quality control centre that is responsible for the optimisation of folding and assembly of newly synthesised secretory proteins and also disposes of any defective ones (Haas and Wabl, 1983; Wiertz *et al.,* 1996). The correct and efficient operation of this machinery is also vital during stress conditions that result in the accumulation of unfolded proteins in the ER. Exposure of cells to adverse conditions results in the up-regulation of ER chaperones and folding enzymes to ensure the correct folding of these accumulating, unfolded proteins (Lee, 1992).

1.5.7. Possible functions of Grp94

While the physiological role of Grp94 has not been well defined, observations that Grp94 expression is up-regulated by conditions that stimulate the accumulation of unfolded proteins in the ER (Lenny and Green, 1991; Bush *et al.,* 1997; Kozutsumi *et al.,* 1988 Wearsch *et al.,* 1998), suggest a chaperone function for Grp94. For example blocking the proteosome, which caused the accumulation of misfolded proteins destined for degradation, resulted in an increase in Grp94 expression (Bush *et al.,* 1997). This was in agreement with the earlier

studies of Kotzumi and co-workers (1988) who were able to show that expression of mutant forms of the influenza virus haemaglutin in simian cells induced the synthesis of Grp94. Tunicamycin, a glycosylation inhibitor also results in accumulation of malfolded proteins with a resultant increase in Grp94 expression (Kozutsumi *et al.,* 1988). Grp94 has also been found in association with a number of proteins and has been implicated in normal protein folding and assembly of proteins within the ER, again suggesting a chaperone function for this protein (Melnick *et al.,* 1992, 1994; Chavany *et al.,* 1996; Muresan and Arvan, 1997; Linnik and Herscovitz, 1998). Grp94 has also been implicated in Ca^{2+} regulation (Cala and Jones, 1994) and it has been observed that the depletion of ER Ca^{2+} stores results in the upregulation of Grp94 (Lee, 1992; Li *et al.,* 1992; Li *et al.,* 1993; Little and Lee, 1995; McCormick *et al.,* 1997; Liu *et al.,* 1998). There is also evidence for a role in antigen presentation, which will be discussed further in section 1.5.9 (Li and Srivastava, 1993; Chavany *et al.,* 1996, Nieland *et al.,* 1996; Blachere *et al.,* 1997; Sastry and Linderoth, 1999).

1.5.8. Grp94 in quality control of the ER

Protein folding

It is known that Grp94 associates with other molecular chaperones found in the ER, namely, Grp78/BiP, calreticulin, calnexin and Hsp47. However, the mechanism of action of these proteins is not clear. Data of Melnick *et al.* (1992, 1994), suggest that Grp94, the most abundant chaperone in the ER lumen, might act by binding proteins that have attained a higher degree of secondary structure after a prefolding step by BiP. In studies using immunoglobulin as a substrate, it was found that BiP preferentially binds an early disulphide intermediate of light chain and Grp94 exclusively binds fully oxidised molecules. These results indicate that Grp94 is itself a chaperone that acts after BiP (Melnick *et al.,* 1994). Sequential co-immunoprecipitation analysis showed that Grp94 can bind directly to immunoglobulin chains rather than indirectly via BiP (Melnick *et al.,* 1994) and it has been proposed that BiP and Grp94 act in tandem on overlapping populations of folding intermediates and that Grp94 assists a more advanced step in folding, perhaps aiding the assembly of immunoglobulin subunits (Melnick *et al.,* 1994). This is similar to the mechanism of action of Hsp90, its cytosolic counterpart, which also appears to bind proteins with a high degree of secondary structure in a complex together with hsp70 and other co-chaperones. Direct interaction of Grp94 with substrate was also demonstrated by studies analysing the interaction of Grp94 with thyroglobulin (Kuznetsov *et al.,* 1994). It was shown that overexpression of Grp94 resulted in thyroglobulin retention within the ER as a direct consequence of complex formation between and apparent thyroglobulin folding intermediate

and this ER chaperone (Muresan and Arvan, 1997). Further evidence for the interaction of Grp94 with substrate came from studies with p185erbB2, a receptor tyrosine kinase. This protein was shown to form a stable complex with Grp94 and a role for Grp94 in p185erbB2 stability has been proposed. The data further suggests that failure of newly synthesised p185erbB2 to associate with Grp94 prevents the translocation of the newly synthesised protein to the plasma membrane, instead trapping it in an intracellular vesicular compartment within the ER (Chavany *et al.,* 1996). Co-immunoprecipitation studies showed that that Grp94 interacts with both early and more advanced folding intermediates of apoB and presumably mediates its folding into a secretion-competent form (Linnik and Herscovitz, 1998). Grp94 has also been shown to accumulate in response to the presence of malfolded proteins in the ER (Kozutsumi *et al.,* 1988) and Bush and co-workers (1997) found that blocking the proteosome resulted in an increase in proteins destined for degradation, this in turn resulting in an increase in Grp94 expression. Tunicamycin, a potent inhibitor of N-glycolsylation of proteins, leads to an accumulation of misfolded proteins in the ER, with a resultant increase in Grp94 expression (Kozutsumi *et al.,* 1988). This reinforces the suggestion of a chaperone function for this protein. Grp94 has also been found to form stable complexes with viral and cellular proteins in the ER. For example, a mutant form of the herpes simplex virus 1 glycoprotein B (gB) with altered conformation, accumulates in the ER. Grp94 complexed with the mutant gB but not with the fully processed viral protein (Navarro *et al.,* 1992). Similarly, major histocompatability complex Class II molecules expressed in the absence of the invariant chain remained in an immature form in the ER and were found to be associated with Grp94 (Schaiff *et al.,* 1992).

Ca^{2+} homeostasis

The experiments mentioned above certainly suggest a role for Grp94 in the folding of proteins within the ER, but it has also been implicated in regulating Ca^{2+} levels within the ER. Grp94, a Ca^{2+}-binding protein (Cala and Jones, 1994), has been proposed to have a role in protecting cells against fluctuating Ca^{2+} levels (Lee, 1992; Li *et al.,* 1992; Li *et al.,* 1993; Little and Lee, 1995; McCormick *et al.,* 1997; Liu *et al.,* 1998). Grp94 shows a high micromolar affinity for Ca^{2+} as compared to Hsp90 (Csermely *et al.,* 1995), thus suggesting a role for Grp94 in calcium regulation. The ER is the major intracellular reservoir of Ca^{2+} (Sambrook, 1990) and a number of vital cellular functions depend on this pool, depletion of which can be highly detrimental to cells (Sambrook, 1990; Short *et al.,* 1993; Vitale and Denecke *et al.,* 1999). These functions include protein processing within the ER (Gething and Sambrook, 1992), maintenance of high translation rates of newly transcribed messages (Brostrom and Brostom, 1990), preserving the structural integrity of the ER (Koch *et al.,* 1986; Booth and

Kock, 1989), and regulating cell proliferation and cell cycle progression (Short *et al.,* 1993). Under physiological conditions the ER Ca^{2+} pool is maintained by a Ca^{2+}-ATPase that pumps Ca^{2+} from the cytoplasm into the ER (Thastrup *et al.,* 1990). During stressful conditions that may result in Ca^{2+} depletion, it has been shown that the ER Ca^{2+} store is mediated in part by the intraluminal Ca^{2+} binding proteins Grp94 (Koch *et al.,* 1986; Mazarella and Green, 1987). Transcription of the genes for these Grp94 and Grp78 is elevated in response to ER Ca^{2+} pool depletion, and in epithelial cells and fibroblasts, Grp78 and Grp94 have been shown to be regulated through common Ca^{2+} responsive promoter elements that respond to ER Ca^{2+} pool depletion (Lee, 1987; Li *et al.,* 1993).

Ca^{2+} pool depletion can be induced by the addition of the Ca^{2+}-ATPase inhibitor, thapsigargin or calcium ionophores such as A23187 (Thastrup *et al.,* 1990). This Ca^{2+} depletion blocks ER protein processing causing partially folded proteins to accumulate, which results in an up-regulation of Grp94 (Kim *et al.,* 1987; Lee, 1987; Lee, 1992; Li *et al.,* 1993; Little and Lee, 1995; Lievremont *et al.,* 1997; McCormick *et al.,* 1997). It was found that increases in expression of Grp94 in cells with depleted Ca^{2+} stores did not result in loss of viability, but this was reversed when Grp94 expression was repressed (Li *et al.,* 1993). This indicates that Grp94 gene induction is a protective response mechanism by which cells accommodate to potentially lethal stress caused by the disruption of intracellular Ca^{2+} homeostasis.

Recently it has been demonstrated that Ca^{2+} is actively involved in protein folding and that Ca^{2+} depletion results in malfolded proteins (Bromstrom and Bromstrom, 1990; Sambrook, 1990; Vitale and Denecke, 1999). In light of these findings, it is possible that Grp94 may play a dual role in retaining calcium as well as refolding of malfolded proteins under conditions of Ca^{2+} depletion.

Proteolytic machinery

Another aspect of quality control in the ER is the disposal of excessively malfolded proteins, which involves their presentation to the proteosome, proposed to be attached to the outer membrane of the ER (Kopito, 1997). Studies with selected targets, such as p185erbB2, a receptor tyrosine kinase, indicate that Grp94 may be involved in presentation of substrates to the proteosome (Chavany *et al.,* 1996). Further evidence for a link between Grp94 and the proteosome arises from the observation that inhibitors specific to the proteosome were able to induce Grp94 expression, whereas other inhibitors such as those of cysteine, serine and metalloproteases had no effect (Bush *et al.,* 1997). Further evidence that Grp94 may be

linked to the proteosome is the reported degradation of highly purified preparations of Grp94 (Srivastava *et al.,* 1986; Anderson *et al.,* 1994).

1.5.9. The role of Grp94 in antigen presentation

In the mid 1980's both human Grp94 and mouse Hsp90 were identified as tumour specific antigens expressed on the surface of various tumour cells (Srivastava *et al.,* 1986; Ullrich *et al.,* 1986). The significance of this is uncertain but it has been postulated that they play a role in antigen presentation, which is supported by their capacity to bind a variety of peptides (Li and Srivastava, 1993; Nieland *et al.,* 1996; Blachere *et al.,* 1997; Sastry and Linderoth, 1999).

Studies show that complexes of Grp94 molecules with a variety of synthetic peptides can be generated *in vitro*; the binding is specific and the complexes are immunologically active (Srivastava *et al.,* 1986; Nieland *et al.,* 1996; Blachere *et al.,* 1997; Wearsch and Nicchita, 1997; Sastry and Linderoth, 1999). It has also been shown that Grp94 can bind a viral peptide and that this complex is capable of eliciting an immune response (Nieland *et al.,* 1996). These observations have led to the suggestion that Grp94 acts as a chaperone for the peptides, i.e it acts as an acceptor for peptides transported to the ER and enables peptide loading of MHC class I (Li and Srivastava, 1993). These are just a few examples of many that implicate Grp94 in antigen presentation.

These examples give an indication of the importance of this protein during normal conditions as well as during stress conditions. However the exact mechanism of action still remains to be determined.

1.6. Molecular characteristics and structure of Hsp90

Despite the fact that Hsp90 is one of the major heat shock protein families, very little is known about the overall structure of the protein and its the mode of action. Molecular analysis of the cytoplasmic Hsp90 has begun to elucidate the structure and possible mechanisms employed by this class of proteins.

The amino acid sequence of Hsp90 was first published in 1984 (Farelly and Finkelstein), but to date relatively little is known about the functional role of various segments of the protein. Sequence alignment and proteolytic digestions indicates that Hsp90 contains two well conserved regions attached to each other by a relatively flexible, highly charged linker loop

(Koyasu *et al.*, 1986; Stebbins, 1997). This linker region is of variable length in different organisms and is nearly completely absent in prokaryotic Hsp90s (Bardwell and Craig, 1987). The native protein exists as an elongated dimer (Figure 1.7) (Rose *et al.*, 1987) and electron microscopy and antibody binding studies have indicated that two Hsp90 monomers are joined in the middle by their C-terminal domains and that the N-terminal domains point in opposite directions (Koyasu *et al.*, 1986; Wearsch and Nicchitta, 1996; Maruya *et al.*, 1999;) The dimer appears to be quite flexible as different shapes of the molecule have been detected. Heat shock and, to a lesser extent, the presence of ATP trigger structural changes in the Hsp90 molecule which result in the interaction of the N-terminal domains of the same chimeric Hsp90 with each other (Maruya *et al.*, 1999).

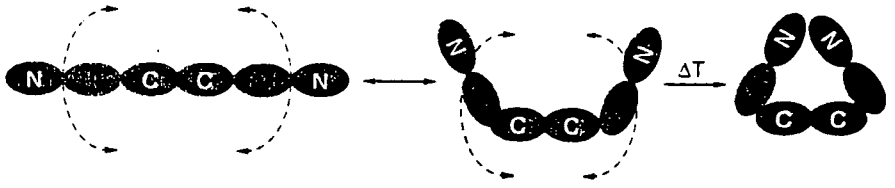

Figure 1.7: Schematic representation of the native dimer of Hsp90 (Buchner, 1999).

Hsp90 is a phosphorlyated dimer (Rose *et al.*, 1987; Lees-Miller and Andersen, 1989a,b; Radanyi *et al.*, 1989; Minami *et al.*, 1991) and dimerisation is required for the vital functions of Hsp90 (Minami *et al.*, 1994). Hsp90 also has a tendency to form oligomers, and non-ionic detergents, heat treatment, divalent cations, ATP and higher Hsp90 concentrations all induce oligomerisation in native Hsp90 (Minami *et al.*, 1991; Minami *et al.*, 1993; Nemoto *et al.*, 1996).

Hps90, like other chaperones, is a hydrophobic protein and its hydrophobicity is increased further after heat shock (Iwasaki *et al.*, 1989; Yamamoto *et al.*, 1991). Hsp90 also contains two highly charged regions, one of which is located in the linker region between the N- and the C-terminal domain and the other within the C-terminal domain. These structures, and possibly the exposed hydrophobic surfaces, probably also have a role in determining the protein binding characteristics of Hsp90 (Binart *et al.*, 1989).

1.6.1. Molecular characteristics and structure of the Hsp90 N-terminal domain

Proteolytic digestions reveal that Hsp90 is made up of 3 domains, a ~25-kDa N- terminal domain, a ~30-kDa linker region and a ~12-kDa C-terminal domain (Stebbins *et al.*, 1997).

The N-terminal domain is highly conserved amongst species (Gupta, 1995), and although the crystal stucture of Hsp90 has not yet been resolved (Prodromou *et al.,* 1997a, b), X-ray crystallography has recently revealed the 3-D structure of the N-terminal domain for both the yeast (Prodromou *et al*, 1997 a, b) and human (Stebbins *et al*, 1997) proteins. The amino acid sequence and tertiary structure of these two domains are extremely similar, consisting of a highly twisted 8-stranded antiparallel β-sheet covered on one face by α-helices resulting in an αβ sandwich (Prodromou *et al.,* 1997a, b; Stebbins *et al.,* 1997; Buchner, 1999). The quaternary structure of the yeast and human Hsp90 N-terminal domains observed in the crystals are different, however, as the yeast preparation was dimeric and the human is monomeric (Prodromou *et al.,* 1997 a, b; Stebbins *et al.,* 1997).

The yeast Hsp90 N-domain crystallizes as a dimer in which the β-sheets at the C-terminal end of each monomer make antiparallel interactions to generate a continuously H-bonded 16-stranded β-sheet. This sheet then folds back on itself to form a cylindrical channel between the two monomers which is 25 Angstrom deep and 8 Angstrom wide (Figure 1.8). The overall structure resembles that of a molecular clamp, comprising a hinge made up of strands at the dimer connection and jaws made up of the concave faces of β-sheets, which act to trap the possible substrates (Prodromou *et al.,* 1997a). The channel is made up of residues highly conserved across species and the channel is large enough to bind a peptide chain in an extended conformation, with little or no steric hindrance (Prodromou *et al.,* 1997a). The N-domain, however is not the main site of dimerization, as *in vivo* it has been shown to be totally dependent on regions in the C-terminal (Minami *et al.,* 1994; Meng *et al.,* 1996) and it has been suggested that the dimer may have been an artifact of the dimerisation process.

Figure 1.8: Representation of the structure of the yeast Hsp90 N-terminal domain (Prodromou et al., 1997a).

The human N-domain crystallizes as a monomer comprising 9 α-helices and an antiparellel β-sheet of 8 strands (Stebbins *et al.,* 1997). No molecular clamp was observed, but at the centre of the helical face of the sandwich a deep pocket of 15 Angstrom extends down

towards the buried β-sheet. The β-sheet forms the base of the pocket with 3 α-helices and a loop making up the walls. Geldanamycin (GA), a potent inhibitor of Hsp90, was co-crystallised with the N-terminal domain of Hsp90 and was found to bind specifically in this pocket region (Stebbins *et al.,* 1997). Residues within the pocket are highly conserved across species and the pocket is thought to be a binding site for segments of polypeptide chains from incompletely folded client proteins (Stebbins *et al.,* 1997). Interestingly, the conserved residues are mainly in and around the pocket, with 82% of the residues lining the interior of the pocket being identical from *E.coli* through to humans. A surface groove leads along the helical face of the sandwich into the pocket. Its shape is not as pronounced as the pocket as it is shallower and broader. This groove is also conserved, although not as well as the pocket. Many of the residues within the groove are polar or charged and they do not have significant roles in maintaining the structural integrity of this domain. Conservation of these residues suggests that the groove may participate in intermolecular interactions important for Hsp90 function.

The N-terminal domain of Hsp90 from these two different species are extremely similar with the exception that the yeast protein forms dimers whereas the human counterpart does not (Prodromou *et al.,* 1997a; Stebbins *et al.,* 1997). The dimer formation may, however, be a consequence of the crystallisation process as the fragment used to crystalise the yeast Hsp90 N-terminal domain was longer and contained more of the C-terminal region than the human Hsp90 N-terminal fragment did (Prodromou *et al.,* 1997a,b). Both domains do possess a pocket that protrudes into the helical face extending down towards the β-sheet. There is a high degree of conservation of residues between species in this pocket region, indicating a functional role for this region. It has been proposed that it may be a possible substrate binding domain (Young *et al.*,1997; Scheibel *et al.*, 1998; Scheibel *et al.*, 1999a) and it has also been shown to be an ATP binding domain (Prodromou *et al.,* 1997a,b; Grenert *et al.,* 1997).

ATP binding

There has been much debate as to whether Hsp90 does in fact bind and hydrolyse ATP (Weich *et al.,* 1992; Johnson and Toft, 1994, 1995; Jakob *et al.,* 1996; Grenert *et al.,* 1997; Stebbins *et al.,* 1997; Scheibel *et al.,* 1997, 1998). However, recent studies have proven unequivocally that Hsp90 does indeed bind ATP (Prodromou *et al.,* 1997b; Obermann *et al.,* 1998). Prodromou and co-workers (1997b) demonstrated that ATP co-crystalises with yeast Hsp90 and that it binds to Hsp90 in the pocket on the helical face of the N-domain. The 15 Angstrom-deep pocket that binds GA in the human Hsp90 N-terminal domain also has a

unique glycine rich ATP binding motif first identified in a bacterial topoisomerase (Bergerat *et al.,* 1997). Crystal structures of the N-terminal domain of human Hsp90 revealed that ATP binds to this domain (Obermann *et al.,* 1998).

Interestingly, it appears that Hsp90 may belong to an emerging ATPase superfamily (GHKL superfamily) (Dutta and Inoye, 2000; Ban *et al.,* 1999). This was realised when an unexpected, yet striking, similarity in overall tertiary structure between the N-terminal domain of Hsp90 and the N-terminal ATP binding domain of the bacterial type II topoisomerase, DNA gyrase B of *E.coli* was observed (Wigley *et al.,* 1991). MutL, a DNA mismatch repair protein and histidine kinases EnvZ and CheA (Ban *et al.,* 1999; Bilwes *et al.,* 1999) have recently been identified as members of this family.

Consistent with the structural similarity between the Hsp90 and DNA gyrase N-terminal domains, there is a remarkable similarity in the conformation of bound ATP and the many of the protein residues that interact with the nucleotide are conserved between the two proteins (Prodromou *et al.,* 1997b). A significant example of this is a conserved glutamate residue in the gyrase protein, E42, which corresponds to E33 in the yeast protein and E47 in the human Hsp90 and this has been identified as the catalytic center for ATP hydrolysis in the gyrase (Jackson and Maxwell, 1993). The implications for ATP binding and hydrolysis will be discussed in detail below (section 1.7.1).

Little is known about the site of interaction with non-native proteins on Hsp90 but it has been shown that the Hsp90 N-terminal domain also has a peptide binding site that seems to bind preferentially unfolded polypeptides. The peptide binding is regulated by ATP binding (Young *et al.,* 1997; Scheibel *et al.,* 1998, 1999a). The N domain pocket has also been suggested to be involved in peptide binding. *In vitro* and *in vivo* studies have shown that the N-domain is capable of preventing aggregation of unfolded proteins and that this activity is regulated by ATP (Young *et al.,* 1998; Scheibel *et al.,* 1998, 1999a).

1.6.2. Molecular characteristics and structure of the Hsp90 linker region

The linker is a highly charged region (Minami *et al.,* 1993) that has been found only to be present in eukaryotes (Bardwell and Craig, 1987). This linker joins the N- and C-terminal domains of the Hsp90 molecule, and its length varies amongst different organisms (Minami *et al.,* 1993). The linker region represents the most striking difference between bacterial and eukaryotic Hsp90s, and it may thus be involved in the gain of function of eukaryotic Hsp90s (Scheibel *et al.,* 1999).

The linker region has been shown to participate in the association of the protein with steroid hormone receptors (Tbarka *et al.*, 1993; Cadepond *et al.*, 1993; Dao-Phan *et al.*, 1997) and with protein kinase CK-II (Miyata and Yahara, 1995). It contains alternating lysine and glutamate residues (referred to KEKE motifs) which are thought to be involved in protein-protein interactions (Realini *et al.*, 1994). Hsp90 is a calcium binding protein (Minami *et al.*, 1993) and part of the KEKE region has two α-helices that show high similarity to calcium binding EF hand structures (Nardai *et al.*, 1996). Two major *in vivo* phosphorylation sites are found within the putative Hsp90 EF hands (Csermely *et al.*, 1998) and can be phosphorylated by protein kinase CK-II, a kinase known to form complexes with Hsp90 (Miyata and Yahara, 1995). The overlap of these major phosphorylation sites with the putative calcium binding sites suggests that phosphorylation of the major phosphorylation sites might be required for calcium to bind to Hsp90 (Csermely *et al.*, 1998).

A bipartite nuclear localisation sequence is located next to the EF-hand-like structures (Nardai *et al.*, 1996). Under normal conditions, this signal appears to be hidden in the interior of the protein, but deletion mutants in which the signal is exposed results in the translocation of the truncated protein to the nucleus (Meng *et al.*, 1996). A small portion of Hsp90 is known to reside in or move to the cell nucleus in resting cells and after heat shock (Collier and Schlessinger *et al.*, 1986) and this sequence may have a role in directing the protein to the nucleus. It has also been proposed to participate in the nucleo-cytoplasmic movement of steroid receptors (Kang *et al.*, 1994).

The charged region has been proposed to have a role in regulating the chaperone activity of the protein. Scheibel and co-workers (1999b) were able to show that the charged region causes the N-terminal domain to have an increased affinity for nonnative proteins and it establishes a crosstalk between peptide and ATP binding.

1.6.3. Molecular characteristics and structure of the Hsp90 C-terminal domain

Although no crystal structures for the ~12-kDa C-terminal domain have been resolved, studies have shown that this region is critically important for Hsp90 dimerisation (Nemoto *et al.*, 1996, 1997; Meng *et al.*, 1996). Dimerisation is an intrinsic property of Hsp90, required for its biological function in intact cells (Minami *et al.*, 1994). The C-terminal domain also contains the common tetratricopeptide (TPR) acceptor site for steroid receptor associated immunophilins (FKBP52, CyP40) and Hop (Chen *et al.*, 1998; Young *et al.*, 1998; Carello *et*

al., 1999). A TPR motif is a 34 amino acid consensus sequence that mediates protein-protein interactions in a range of cellular pathways (Goebl and Yanagida, 1991) and many of the Hsp90 associated cofactors contain multiple copies of this TPR motif (Nair *et al.,* 1996; Frydman and Hohfeld, 1997; Pratt *et al.,* 1997). For example, the binding of FKBP52, CyP40 and protein phosphatase pp5 to Hsp90 is mediated by their TPR-containing domains and the binding of p60 to Hsp90 requires a central region of p60 that contains 5 TPR motifs (Chen *et al.,* 1996; Owens-Grillo *et al.,* 1996; Fryman and Hohfeld, 1997).

The C-terminal fragment has also been shown to possess chaperone activity, independent of the N-terminal domain (Young *et al.,* 1997; Scheibel *et al.,* 1998, 1999a). The C-terminal fragment binds to partially folded peptides in an ATP-independent way presumably regulated by co-chaperones that bind in the same region (Scheibel *et al.,* 1998). Thus the N-terminal domain interacts selectively with completely unfolded proteins and small peptides in an ATP-dependent manner, whereas the C-terminal fragment appears to act as a promiscuous binding site for more structured substrates (Young *et al.,* 1997; Scheibel *et al.,* 1998).

The presence of chaperone and dimerisation functions within the C-terminal domain which is also responsible for mediating interactions with TPR co-chaperone proteins is interesting. It is possible that the Hsp90 TPR containing partner proteins may have an important modulating role in Hsp90 function.

1.7. Hsp90 mechanism of action

1.7.1. An ATPase molecular clamp.

Whether Hsp90 itself binds and hydrolyses ATP has been an extremely controversial issue (Wiech *et al.,* 1992; Csermely *et al.,* 1993; Johnson and Toft, 1994; 1995; Jakob *et al.,* 1996; Grenert *et al.,* 1997; Scheibel *et al.,* 1997, 1998). However, crystal structures of the N-terminal domains of both the yeast and human Hsp90 have unequivocally demonstrated ATP binding to this domain (Prodromou *et al.,* 1997b; Obermann *et al.,* 1998). Higher resolution methods using spin-labelled conformational probes confirmed ATP binding to Hsp90, even though it was with low affinity (Grenert *et al.,* 1997; Scheibel *et al.,* 1997). The question of ATP hydrolysis has also been addressed, and biochemical studies have shown that Hsp90 hydrolyses ATP *in vitro* (Obermann *et al.,* 1998; Panaretou *et al.,* 1998; Scheibel *et al.,* 1998). These studies indicate that Hsp90 is in fact an ATP-dependent molecular chaperone and ATP binding and hydrolysis have been shown in further studies to be

essential to *in vivo* functioning of the protein (Obermann *et al.,* 1998; Grenert *et al.,* 1999; Panaretou *et al.,* 1999; Prodromou *et al.,* 1999; Prodromou *et al.,* 2000).

Although it has been proven that Hsp90 binds and hydrolyses ATP, how this ATPase activity regulates Hsp90 function and interaction with polypeptide substrates is not yet understood. The structure of the N-terminal domain of Hsp90 resembles the ATP binding sites in GyrB (Wigley *et al.,* 1991), MutL (Ban *et al.,* 1999) and histidine kinases (Bilwes *et al.,* 1999). Residues that interact with ATP within the ATP binding sites of Hsp90 and the proven ATPAse DNA gyrase B are conserved. Importantly, the catalytic glutamate (Glu-42) responsible for the ATPase activity of gyrase B (Jackson and Maxwell, 1993) is conserved in the N-domain of Hsp90 (Glu-33). This indicates that Hsp90 is equipped for an ATPase activity with essentially the same catalytic mechanism as DNA gyrase B (Prodromou *et al.,* 1997b).

The ATPase proteins belonging to this family are innately dimeric and are joined by their C-terminal domains. The dimerisation occurs independently of the nucleotide status of the N-terminal domain. ATP binding induces dimerisation of the N-termini resulting in an opening and closing motion of a 'molecular clamp' which is hinged at the C-terminus (Ban *et al.,* 1999; Kampranis *et al.,* 1999). Prodromou and co-workers (1997b) have shown that Hsp90 has a similar mechanism of action. Bound ATP promotes the association of the N-terminal domains within the Hsp90 dimer, and subsequent ATP hydrolysis is highly cooperative and dependent on that dimerisation. These properties together with the inherent dimerisation at the C-terminus, describe a molecular clamp whose opening and closing by transient dimerisation of the N-terminals is directly linked to the ATPase cycle (Prodromou *et al.,* 1997b)

Dimerisation of the N-termini, however, has been a subject of dispute. The crystal structure of the N-terminal domain of yeast Hsp90 revealed a dimer interface formed by the association of C-terminal strands in an antiparrallel β-sheet (Prodromou *et al.,* 1997a). On the other hand, the crystal structure of the N-terminal domain of human Hsp90, in which this C-terminal strand was not included, is monomeric. The plausibility of N-terminal dimerisation was also contested by EM studies which showed a divergent structure for the Hsp90 dimer (Koyasu *et al.,* 1986; Wearsch and Nicchitta, 1996) in which only the C-termini are associated. Additionally, the N-terminal domains are not able to form stable dimers in solution (Scheibel *et al.,* 1998). In support of the possibility of N-terminal dimerisation however, significant conformational rearrangement of Hsp90s was detected by electron

microscopy upon incubation of Hsp90 at high temperature and after addition of ATP *in vitro* (Maruya *et al.,* 1999). Under these conditions, the N-terminal domains of the Hsp90 dimer that usually point in opposite directions, move closer to each other. Crosslinking experiments also showed that the N-terminal domains of an Hsp90 dimer were close together after the addition of ATP (Prodromou *et al.,* 2000). Despite the lack of direct evidence, it is plausible that structural rearrangements that bring the 2 N-terminal domains together, such as ATP binding, can occur during the Hsp90 chaperone cycle (Weikl *et al.,* 2000).

The study of Prodromou *et al.* (2000) shows that Hsp90 is related to GyrB and MutL in sharing a common structure in the N-terminal dimerisation interface and an essentially identical ATPase coupled mechanism (Prodromou *et al.* 2000). Despite this similarity, these proteins have distinct biological functions and participate in different molecular systems, interacting with different components. For example the molecular clamp of DNA gyrase B is involved in the binding and release of a DNA duplex during the unwinding reaction. The clamp binds ATP, closes around the DNA, and positively charged residues lining the clamp interact with the phosphate backbone of the DNA during the reaction (Wigely *et al.,* 1991; Kampranis *et al.,* 1999). In a similar manner, the 'jaws' of the molecular clamp may be the site of interaction of non-native proteins with Hsp90 (Prodromou *et al.,* 2000). Although *in vitro* and *in vivo* studies (Scheibel *et al.,* 1998, 1999a; Young *et al.,* 1997) have shown that the N-terminal domain is able to bind unfolded polypeptides and prevent aggregation, the site for polypeptide binding on Hsp90 is still not known, so this remains as speculation.

These studies have provided evidence for a molecular clamp function of Hsp90 that is driven by the ATPAse cycle, but how does this activity relate mechanistically to substrate binding and release? A series of biochemical studies and analysis of deletion mutants (Prodromou *et al.,* 1997b, 1999, 2000; Obermann *et al.,* 1998; Grenert *et al.,* 1999; Hartl and Young *et al.,* 2000; Weikl *et al.,* 2000) have begun to put the pieces of the puzzle into place. From this, a proposed model for the ATPase driven substrate-binding cycle of Hsp90 has emerged (Figure 1.9).

Substrate binds to a nucleotide-free form of Hsp90 (Prodromou *et al.,* 1999, Hart and Young, 2000), after which ATP binds to Hsp90 (Hartl and Young, 2000). The binding of ATP is a rapid, but weak reaction (Weikl *et al.,* 2000). As a consequence of ATP binding, the Hsp90 molecule undergoes a conformational change (Sullivan *et al.,* 1997; Grenert *et al.,* 1999; Weikl *et al.,* 2000) effectively trapping the ATP molecule in the binding pocket, thus

committing it to hydrolysis (Weikl *et al.,* 2000). This conformational trapping of the ATP molecule requires the complete nucleotide binding cleft of the N-terminal domain and regions of the Hsp90 molecule located in its C-terminal domain (Prodromou *et al.,* 1997b; Weikl *et al.,* 2000). The C-terminal domain binds the TPR containing co-chaperone proteins which have been shown to regulate ATPase activity of Hsp90 (Prodromou *et al.,* 1999) thus indicating that the C-terminal region is important in regulating the function of the protein. It was shown that the yeast Hop homologue inhibits ATPase activity and it was able to displace GA bound to the ATP binding domain. These results suggest a direct interaction between Hop and the ATP binding site of the N-terminal domain (Prodromou *et al.,* 1999). This again supports the possibility that conformational changes cause rearrangement of different regions of the protein to regulate its function during different parts of the cycle of its function.

Consistent with the idea of ATP induced conformational changes, it has been reported that Hsp90 becomes more hydrophobic in the presence of ATP (Sullivan *et al.,* 1997) and as mentioned above, EM studies show that ATP brings the N-termini of an Hsp90 dimer together (Maruya *et al.,*1999). It has been proposed that ATP binding induces N-terminal dimerisation within an Hsp90 dimer (Prodromou *et al.,* 2000) as is seen in its structurally related counterparts GyrB and MutL (Kampranis *et al.,* 1999; Ban *et al.,* 1999). This N-terminal dimerisation enhances ATP hydrolysis in Hsp90 (Prodromou *et al.,* 2000) and is also believed to allow the binding of p23 to Hsp90 (Prodromou *et al.,* 1997b; Obermann *et al.,* 1998; Grenert *et al.,* 1999; Weikl *et al.,* 2000). p23 has been shown to bind selectively to the dimerised conformation of Hsp90 that is induced by ATP binding (Prodromou *et al.,* 1997b; Obermann *et al.,* 1998; Grenert *et al.,* 1999). The actual location of the p23 binding site on Hsp90 as well as the stoichiometry of the reaction is not known (Prodromou *et al.,* 1997b; Weikl *et al.,* 2000), but it is known that The N-terminal ATPase and the C-terminal dimerisation sequence of Hsp90 are both required for binding (Chen *et al.,* 1998).

After ATP binding, the conversion of Hsp90 to the ADP state through hydrolysis of the bound ATP is responsible for the fast release of substrate and p23 has been proposed to increase the efficiency of this release reaction (Grenert *et al.,* 1999; Hartl and Young, 2000). Again, studies have shown that the ATPase activity of Hsp90 is dependent on the dimeric state of the protein as the process was shown to occur more readily in the already dimeric state of Hsp90 (Weikl *et al.,* 2000). Activity is also dependent on regions throughout the molecule as deletion mutants show decreased ATPase activity (Prodromou *et al.,* 1997b, 1999, 2000; Obermann *et al.,* 1998; Weikl *et al.,* 2000). This supports the probability of conformational

changes induced by ATP binding that result in the alignment of regions of the molecule to increase the efficiency of ATP hydrolysis. ATP hydrolysis represents the rate limiting step of the overall ATPase cycle (Weikl *et al.*, 2000). The ATPase cycle is completed by the release of ADP, after which Hsp90 returns to its nucleotide-free state (Hartl and Young, 2000; Weikl *et al.*, 2000). It has been proposed that the co-chaperone, Hop, stabilises this nucleotide-free form of Hsp90 temporarily to allow a fresh substrate protein to be loaded by a mechanism involving Hop and Hsp70 (Prodromou *et al.*, 1999).

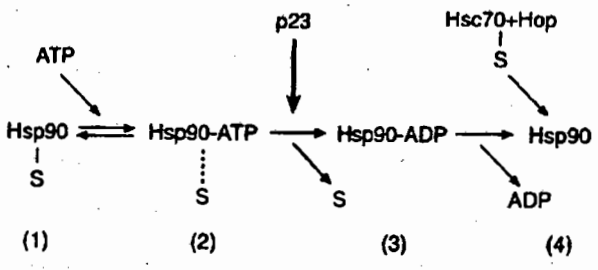

Figure 1.9: ATP-driven substrate binding cycle of Hsp90. (1) Substrate protein(s) is stably bound by the nucleotide-free state of Hsp90. (2) Binding of ATP onto Hsp90 results in slow release of substrate. (3) Conversion of Hsp90 to the ADP state through ATP hydrolysis produces fast and complete release of substrate, enhanced by p23. (4) Substrate may be loaded onto Hsp90 at the nucleotide-free state via Hsc70 (Hsp70) and Hop (Young and Hartl, 2000).

An important property of this system is that ATP hydrolysis is responsible for the efficient release of substrate (Hartl and Young, 2000). As mentioned above, Hsp90 has recently been classified as a member of a newly emerging ATPase family of proteins (Bergerat *et al.*, 1996, Ban and Young, 1998; Ban *et al.*, 1999; Dutta and Inoye, 2000). The model that has been proposed for the ATPase cycle of Hsp90 agrees with the biochemical mechanism of these proteins (Hartl and Young, 2000). ATP binding to GyrB results in homodimerisation of the ATPase domain forming a clamp on the DNA strand. Hydrolysis of the bound ATP results in the release of the DNA and resetting of the enzyme (Berger *et al.*, 1996; Kampranis *et al.*, 1999). The mechanism of action of MutL is similar as ATP binding induces homodimerisation and hydrolysis opens the protein by separating the dimer (Ban *et al.*, 1999). It is therefore reasonable to assume that Hsp90 may follow a similar cycle, in which the ATP-bound form of the chaperone clamps around a substrate polypeptide and subsequent hydrolysis results in release of the trapped substrate (Hartl and Young, 2000). These conformational changes may also allow for optimisation of the molecule to perform different functions through the rearrangement of different regions of the protein. A similar

situation exists in the case of GyrB and the signalling molecule, CheA. Binding and hydrolysis of ATP induces conformational changes that are tightly linked to their topoisomerase and signalling reactions, respectively (Kampranis *et al.,* 1999; Bilwes *et al.,* 1999).

In this ATPase cycle, p23 has been suggested to act to facilitate substrate release by linking it to the ATPase cycle of Hsp90. This release factor activity is not absolutely required for Hsp90 function as some dissociation from substrate is observed in the absence of this co-chaperone. It may thus serve to improve the efficiency of Hsp90 function (Hartl and Young, 2000). Hsp90 recognises a wide range of polypeptides and is involved in diverse cellular activities, many of which are still to be understood. The ATPase-driven substrate binding cycle of the Hsp90 is a common theme in these activities (Hartl and Young, 2000). There is however, much to learn about this chaperone, there are other regions of the protein yet to be determined that may reveal more clues to the function, regulation and interaction of this chaperone with its diverse client proteins.

1.8. Molecular characteristics and structure of Grp94

Grp94 shares 50% identity with the cytosolic Hsp90 (Gupta, 1995) and can be subdivided into domain fragments corresponding to those of Hsp90 (Young *et al.,* 1997; Csermely *et al.,* 1998) and thus it follows that these proteins are likely to share many functional characteristics. Very little is known about the structure and molecular characteristics of Grp94 and most of what is known is based on data available for Hsp90 (Csermely *et al.,* 1998).

Grp94, like Hsp90, exists as dimers of identical subunits (Wearsch *et al.,* 1996; Wearsch and Nicchitta, 1996; Nemoto *et al.,* 1996). Rotary-shadowing electron microscopy has revealed that Grp94 dimers show a trinodular elongated rod-like shape (Koyasu *et al.,* 1986; Wearsch and Nicchitta, 1996b) which is similar to that seen for Hsp90. Like Hsp90, dimerisation of Grp94 is promoted by hydrophobic interactions resulting in two Grp94 molecules interacting at their C-terminal ends with the N-terminal domains facing outwards (Wearsch and Nicchitta, 1996). The level of *in vitro* oligomerisation of Grp94 is not as high as that observed for Hsp90 (Nemoto *et al.,* 1996), but *in vivo* the high protein concentration within the ER lumen and the resulting molecular crowding will probably lead to the appearance of Grp94 oligomers. In accordance with this, the formation of tetramers and higher order oligomers following heat treatment was found to be highly dependent on Grp94

concentration (Wearsch and Nicchitta, 1998). Interestingly, Grp94 is able to form a heterodimer with Hsp90 (Nemoto *et al.,* 1996), indicating the importance and evolutionary conservation of dimerisation within the 90-kDa chaperone family (Csermely *et al.,* 1998).

Grp94 is phosphorylated by numerous kinases, including CK-II (Cala and Jones, 1994; Csermely *et al.,* 1995) as is Hsp90. CK-II phosphorylates Grp94 in the middle highly charged region and at 4 C-terminal threonine residues (Cala and Jones, 1994). Also like Hsp90, Grp94 is a hydrophobic protein (Csermely *et al.,* 1998) and it associates with numerous other proteins, such as protein kinases (Cala and Jones, 1994; Csermely *et al.,* 1995; Truijillo *et al.,* 1997), actin filaments and calmodulin (Koyasu *et al.,* 1986, 1989) as well as other ER molecular chaperones such as grp78 (BiP) (Melnick *et al.,* 1992), calreticulin and calnexin for example (Tatu and Helenius, 1997). The ER chaperone complex is not as well described as that of the Hsp90 chaperone complex, but due to the high protein concentration of the ER lumen (~100mg/ml) it can be assumed that Grp94 might be part of an even more complex machinery (Csermely *et al.,* 1998).

Grp94 is a calcium binding protein (Koch *et al.,* 1986; Cala and Jones, 1994) and like Hsp90 it contains EF-hand structures which may serve as calcium binding sites (Csermely *et al.,* 1995). Four high affinity and approximately ten low affinity calcium binding sites have been identified (Van *et al.,* 1989; Hubbard and McHugh, 1996).

One difference between Hsp90 and Grp94 is that Grp94 is a glycoprotein (Csermely *et al.,* 1998). Under normal conditions, this protein is N-glycosylated at Asn-196 (Qu *et al.,* 1994) and interestingly O-glycosylation has also been reported (Poola and Lucas, 1988; Hayes *et al.,*1994; Poola and Kiang, 1994). O-glycosylation is an important modification with regulatory implications, frequently involved in an interdependent relationship with phosphorylation (Hart, 1997). It may therefore play an important role in regulating Grp94 function (Csermely *et al.,* 1998). As with Hsp90, the issue of ATP binding has been a controversial one. However, it has been shown that Grp94 is an ATP binding protein (Li and Srivastava, 1993; Csermely *et al.,* 1995; Rosser *et al.,* 2000) and ATP binding has been shown to lead to autophosphorylation of the protein as well as an ATPase activity (Li and Srivastava, 1993). The N-terminal domain of Grp94 is predicted to have a tertiary structure similar to that of Hsp90 (Gerloff *et al.,* 1997). If this is the case it may contain an ATP binding site as well as a binding site for GA similar to that in Hsp90 (Csermely *et al.,* 1998). This is very likely as GA is known to affect the function of Grp94 in a similar manner to that of Hsp90 (Chavany *et al.,* 1996).

The C-terminal domain of Grp94 contains the region responsible for dimer formation (Nemoto *et al.,* 1996; Wearsch and Nicchitta, 1996b). This domain also harbours a KDEL sequence, which is the common signal for retention of proteins within the ER (Sorger and Pelham, 1987).

1.9. Grp94 mechanism of action

At present the mechanism, site and regulation of peptide binding to Grp94 are unknown. However, studies analysing the binding of peptide to Grp94 using VSV8, the immunodominant peptide epitope of the vesicular somatis virus (VSV), as a substrate, have begun to elucidate the structural basis of the peptide binding reaction (Wearsch *et al.,* 1998).

These results revealed that VSV8 binding was stimulated by chemical dentauration/renaturation of Grp94 and it was stimulated to a greater extent by heat shock (Wearsch *et al.,* 1998). Studies with the environmental-sensitive flourophores, acrylodan and Nile Red, indicated that the activation of Grp94-peptide binding occurred together with a stable, tertiary conformational change which results in enhanced peptide and solvent accessibility to hydrophobic binding sites (Wearsch *et al.,* 1998).

These results are consistent with a two-stage model for peptide binding to Grp94, this is similar to that proposed for Hsp90 by Stebbins and co-workers (1997). Two alternate conformations of the N-terminal domain, referred to as the "open" and "closed" conformations, were identified. It is only in the "open" conformation that the binding pocket is accessible, leading the authors to suggest that conformational conversions between the two forms are likely to have an important role in the regulation of Hsp90 function. In the model proposed for Grp94, the protein exists in either of two conformations, "open" or "closed". Conversion from the closed to the open conformation would require a conformational change, reflecting that proposed by Stebbins *et al.* (1997). However, as for Hsp90 the site or sites of peptide interaction with Grp94 are still unkown and must await direct mapping studies of stable-domain complexes (Wearsch *et al.,* 1998).

Further studies to understand the molecular basis of Grp94-substrate binding have revealed that polypeptide binding to the nucleotide binding domain of Grp94 actually activates its molecular chaperone activities (Wassenberg *et al.,* 2000). Flourophore binding assays have led to the proposal of a model in which ligand binding to the N-terminal nucleotide binding

domain of Grp94 causes a conformational change converting Grp94 from an inactive to an active conformation (Wassenberg *et al.,* 2000). Molecular studies of Hsp90 function have consistently shown a strong link between conformational state and activity. For example heat shock has been demonstrated to elicit a conformational change accompanied by the activation of molecular chaperone activity (Yonehara *et al.,* 1996) peptide binding activity (Blachere *et al.,* 1997; Wearsch and Nicchitta1997) and oligomerisation state (Wearsch *et al.,* 1998).

This is reflected in the case of Grp94 in which the conformational state of the molecule regulates its molecular chaperone and peptide binding activities (Wearch *et al.,* 1998; Wassenberg *et al.,* 2000). Ligand binding to the conserved N-terminal ATP binding domain can regulate the conformation and activity state of Grp94 (Wassenberg *et al.,* 2000). This appears to be an important means of regulation as a ligand mediated regulation of Grp94 conformation would allow the activation of Grp94 chaperone activity under conditions other than heat shock that disrupt protein folding (Wassenberg *et al.,* 2000).

The model that has thus been proposed for Grp94 is that it exists in two conformations. One state displays low chaperone activity. However, during heat shock or in the presence of suitable ligand, Grp94 undergoes conformational changes accompanied by the activation of chaperone activity (Wearsch *et al.,* 1998; Wassenberg *et al.,* 2000).

The mechanism responsible for regulating interaction of Grp94 with substrate is currently under investigation. It has been proposed that Grp94 like Hsp90 follows an ATP-hydrolysis reaction that is linked to substrate binding and release (Prodromou *et al.,* 1997; Obermann *et al.,* 1998; Panaretou *et al.,* 1998; Scheibel *et al.,* 1998). However, this remains to be determined as it has been shown that although the relevant structural components of the adenosine nucleotide binding pocket are conserved between the two proteins, the ligand specificities of the two binding sites differ (Rosser *et al.,* 2000). Unlike Hsp90, Grp94 displays a specific, high affinity binding interaction with the substitute adenosine derivative, *N*-ethylcarboxamidoadenosine. Grp94 was also shown to bind ATP, dATP, AMP and cAMP. It has been suggested that the specificity of ligand binding to the N-terminal adenosine-nucleotide pocket is influenced by domains C-terminal and perhaps N-terminal to the binding pocket. This is possible as sequence divergence is evident in these regions. This has led the authors to suggest that Grp94 may be regulated by adenosine ligands other than ATP or ADP (Rosser *et al.,* 2000).

Evidence in support of ATP binding and ATPase activities is controversial, as it once was for Hsp90, and a general agreement regarding the molecular basis of an adenosine-nucleotide mediated regulation of Grp94-substrate interactions has yet to emerge (Li and Srivastava, 1993; Csermely *et al.,* 1995; Jakob *et al.,* 1996; Wearsch and Nicchitta, 1997).

As can be seen from this section the information available on Grp94 regulation and function is rather limited and fragmentary. Much of what has been determined is based on its similarity to Hsp90. There does seem to be a degree of similarity in function between the two homologues in that conformational changes govern their activity, however it remains to be determined how far the functional similarity extends. The dependence on ATP binding and hydrolysis for function of Grp94 also remains to be determined.

1.10. Hsp90 and Grp94 in plants

1.10.1. Hsp90

There have been few reports on this important class of proteins from higher plants (Boston *et al.,* 1996). However, genes encoding Hsp90s have been reported in a number of plant species including maize (Marrs *et al.,* 1993), tomato (Koning *et al.,* 1992), *Pharbitis nil* (Felsheim and Das, 1992), *Brassica napus* (Krishna *et al.,* 1995) and *Arabidopsis thaliana* (Conner *et al.,* 1990; Takahashi *et al.,* 1992; Yabe *et al.,* 1994; Milioni and Hatzopoulos, 1997; Krishna and Gloor, 2001). Their expression patterns vary in a tissue-specific manner and not all are regulated by heat. This is dealt with at length in Chapter 3 (section 3.1.1).

1.10.2. Grp94

Like Hsp90, Grp94 has not been studied in detail in plants, but it has been identified in barley (Walther-Larsen *et al.,* 1993; Denecke *et al.,* 1993), bean (D'Amico *et al.,* 1992), maize (Shroeder *et al.,* 1993; Boston *et al.,* 1996), periwinkle (Schroeder *et al.,* 1993) and tobacco (Denecke *et al.,* 1993, 1995). Expression studies have revealed that Grp94 is induced by stresses that result in increased protein trafficking through the secretory system (Walther-Larsen *et al.,* 1993) and in stresses that result in accumulation of malfolded proteins (D'Amico *et al.,* 1992). This is dealt with at length in Chapter 3 (section 3.1.2). As Hsp90 proteins have not been examined in great detail in plants as yet, it is understandable that potential substrates for Hsp90 in plants have also not been identified (Boston *et al.,* 1996). However homologues of the chaperone complex have been found in plants and these proteins are functionally conserved between plants and mammals.

1.10.3. Hsp90 chaperone complex in plants

As mentioned earlier, Hsp90 is able to act independently or as part of a chaperone complex. Components of the Hsp90 chaperone complex have been identified in plants (Stancato *et al.*, 1996; Miernyk, 1999; Pratt *et al.*, 2001). Hsp90, Hsp70 and an FKBP-type prolyl-isomerase have been identified as components of the Hsp90 chaperone complex in wheat (Reddy *et al.*, 1998) and other components of the chaperone complex, including, an Hsp40 homologue and a cyclophilin-type prolyl isomerase have been identified in maize (Miernyk, 1999). A Hop homologue has also been identified in soyabean high and high molecular weight immunophilins have been found in a number of plant species (Pratt, 2001). This plant based Hsp90 chaperone machinery is similar but not identical to that of animal cells (Stancato *et al.*, 1996; Miernyk *et al.*, 1999; Pratt *et al.*, 2001) and studies using wheat germ lysate proved that the plant Hsp90 machinery is capable of activating the mammalian glucocorticoid receptor (Hutchinson *et al.*, 1995; Stancato *et al.*, 1996; Krishna *et al.*, 1997). Further studies have also shown that the plant Hsp90 is able to function as part of the mammalian Hsp90 chaperone complex (Dittmar *et al.*, 1998). This conservation of function is reflected in the high degree of sequence similarity shared by Hsp90s from other organisms, which is in the range of 70% from bacteria through to man (Gupta, 1995; Pratt *et al.*, 2001). The co-chaperones that interact with Grp94 at this stage are unknown. It does, however interact with Grp78/BiP the ER homologue of Hsp70 which has been identified in plants (Schoffl *et al.*, 1998).

The first line of evidence for the presence of this chaperone complex in plants arose from the work of Schena *et al.* (1991). They were able to show that all the conditions for steroid regulation, including steroid binding to the receptor, exist in plant cells using a glucocorticoid-dependent gene expression system. Tobacco cells expressing glucocorticoid receptor were able to activate a reporter gene linked to glucocorticoid response elements after treatment of the plant cells with glucocorticoid. This suggested that plants have a similar system that assembles the receptor into a heterocomplex and that binding of plant Hsp90 to glucocorticoid results in a high efficiency binding conformation (Schena *et al.*, 1991). This work, together with experiments that demonstrate stable heterocomplex formation between mammalian glucocorticoid receptor and plant Hsp90 (Stancato *et al.*, 1996; Krishna *et al.*, 1997), set the stage for further elucidation of the Hsp90 chaperone machinery in plants using glucocorticoid receptor as a surrogate substrate in the plant chaperone machinery.

Plant and animal Hsp90s appear to be interchangeable (i.e. they are able to interact functionally with the other components of the complex) and are able, at least in part, to substitute for each other in the activation of glucocorticoid hormone receptor (Stancato *et al.,* 1996). Purified recombinant *B. napus* Hsp90 was shown to have nearly the same activity as purified rabbit brain Hsp90 in an assembly consisting of purified rabbit Hsp70/Hsp40 and human Hop and p23 (Dittmar *et al.,* 1997). Hsp90 purified from wheat germ and from rabbit reticulocytes show partial complementarity, in that a receptor-Hsp90 complex is formed but the receptor is not converted to the steroid binding conformation (Stancato *et al.,* 1996). Studies with cell free lysates reveal that rabbit Hsp90 is able to interact with components of the plant Hsp90 chaperone complex to promote Hsp90-receptor complexes and similar results were obtained when wheat Hsp90 was added to reticulocyte lysate (Stancato *et al.,* 1996). Thus an essential chaperone from the plant kingdom has conserved the ability to interact with the co-chaperones of the animal kingdom and to co-operate with them in the protein folding machinery. There are, however, differences between the two systems. The dynamics of the assembly-disassembly process in the wheat germ extract are different from those in reticulocyte lysates. The glucocorticoid receptor-Hsp90 heterocomplex assembly rates are similar for the two systems, but the heterocomplex disassembly is much faster in wheat germ extract (Stancato *et al.,* 1996). This rapid disassembly has been postulated to be due to the absence of a wheat p23 homologue (Pratt *et al.,* 2001). This finding was supported by an experiment in which the addition of purified human p23 was able to stabilise glucocorticoid receptor-Hsp90 complexes (Hutchinson *et al.,* 1995). This finding is interesting as, to date, no p23 homologue has been identified in plants (Pratt *et al.,* 2001). However, the results above indicate that although receptor heterocomplex formation in animal lysates and in the plant lysate differ in the dynamics of complex assembly, both systems produce a functional system that binds steroid (Pratt *et al.,* 2001).

The fact that components from such different organisms as animals and plants can interact and function together, supports the idea that the function of the complex in protein folding is basic, essential and highly conserved (Pratt *et al.,* 2001). Plant homologues of the mammalian steroid hormone receptors have not yet been reported, and the native targets for this chaperone complex function in these cells are at this time unknown (Miernyk *et al.,* 1999). It has been proposed to have a role in activation of receptors for plant hormones and growth factors and therefore function in a similar manner to its mammalian counterpart (Pratt *et al.,* 2001).

1.11. Aims of this dissertation

This work forms part of a research effort directed at understanding the methods employed by *X. viscosa* to withstand desiccation. Several cDNA clones were isolated from a cDNA library and differential screening revealed that their expression was upregulated during desiccation (Ndima *et al.*, 2001). One of these encoded a protein that showed significant homology with Hsp90 and Grp94.

The aim of this work was the molecular characterisation of the gene through sequencing and comparison with *Hsp90* and *Grp94* homologues. The expression patterns of the putative *X. viscosa Grp94* transcript and protein were to be investigated using northern and western analysis, respectively. Another aim was to express the protein in *E. coli* and purify it for functional analysis *in vitro* and antibody production. It is hoped that this work will contribute to the understanding of desiccation tolerance in *X. viscosa* with a view to the production of transgenic crops with increased drought tolerance.

CHAPTER TWO

GENETIC ANALYSIS OF *XVGrp94* FROM *Xerophyta viscosa*

SUMMARY

A putative XVGrp94, previously isolated from a cDNA library constructed from mRNA extracted from leaves of dehydrated *Xerophyta viscosa*, was found to be truncated at the 5`terminus. The SMART™ RACE kit was used for isolating the full-length *XVGrp94* cDNA. The SMART™ RACE procedure uses the template-switching activity of Moloney murine leukemia virus (MMLV) reverse transcriptase to incorporate a RACE primer sequence into single-stranded cDNA during first strand synthesis. Following reverse transcription, PCR was performed using a universal forward primer and a gene specific reverse primer. The resultant PCR product was cloned and sequenced. Sequence analysis revealed that the cDNA potentially encoded a protein of 812 amino acids with a calculated size of 92.83 kDa. Alignments showed the predicted protein sequence shared a high degree of similarity with other Hsp90s from plants, with an overall identity of 85.5% at the amino acid level. The sequence contained a highly hydrophobic region in the N-terminal domain which is a putative eukaryotic secretory signal, as well as the endoplasmic reticulum (ER) targeting and retention signal, KDEL (Lys-Asp-Glu-Leu), and the Hsp90 protein family signature, NKDIFL (Asn-Lys-Glu-Ile-Phe-Leu). Residues that interact with ATP within the N-terminal domain of Hsp90s are conserved in the N-domain of the putative *X. viscosa* XVGrp94, indicating that it is potentially capable of ATP binding and hydrolysis. Southern blot analysis confirmed the presence of the *XVGrp94* gene within the *X. viscosa* genome and the results, although inconclusive, suggest the possibility of closely related genes.

2.1. INTRODUCTION

A cDNA library was originally constructed from mRNA extracted from *Xerophyta viscosa* leaves dehydrated to relative water contents of 85%, 37% and 5% (Mundree *et al.*, 2000). Differential screening of the cDNA library resulted in the identification of a number of genes that were up regulated during dehydration (Ndima *et al.*, 2001), one of which was a putative ER-located Hsp90. A cDNA represents a single expressed gene, which may be part of a family of similar genes that are expressed under different conditions or at different stages of development. Hsp90 in maize (Marrs *et al.*, 1992) and tomato (Koning *et al.*, 1992) are

examples of Hsp90s that are developmentally regulated. In the case of the tomato Hsp90 (Hsp80), homologues were found at two loci and one was identified as Hsc80 (Heat Shock Cognate 80), an isoform of Hsp90 that is developmentally regulated. The other gene was only partially sequenced and was thought to encode a second Hsp80. This protein may be differentially regulated and could perform different functions to that of Hsc80.

The Hsp90 identified in *Brassica napus* also belongs to a multi-gene family and it was shown to respond to cold and heat and is also developmentally regulated (Krishna *et al.*, 1995). The authors analyzed only one transcript, but it may be that the probe hybridised to more than one transcript and that the expression patterns observed are from more than one homologue. The Hsp90 (Hsp83) identified in *Pharabitis nil* was shown to be a member of a multi-gene family and is present in six to seven copies in the genome. Hsp83A is the most heat-inducible protein in cotyledons of the plant, whereas the other homologues exhibit a low level of heat induction in cotyledons. One member of the family, Hsp83B, is constitutively expressed in the cotyledons of plants grown in continuous light (Felsheim and Das, 1992).

The *Arabidopsis thaliana* genome contains at seven *Hsp90* genes, and the deduced amino acid sequences show that the members can be divided into two groups. Four of them comprise the cytosolic Hsp90 type (AtHsp90-1, AtHsp90-2, AtHsp90-3 and AtHsp90-4) and the remaining three (AtHsp90-5, AtHsp90-6, AtHsp90-7) are predicted to reside within the plastidial, mitochondrial and endoplasmic reticulum compartments, respectively. The occurrence of multiple cytoplasmic AtHsp90 proteins and of family members in other compartments of the cell suggests a range of specific functions and target polypeptides. (Krishna and Gloor, 2001). Sequence comparison shows that there is high homology among the cytosolic members, while there is less homology among the organelle members. Differential gene expression was observed for these homologues. The mRNA of the cytosolic members increased during heat shock whereas the organelle homologues had different gene expression profiles at the temperatures tested. However, treatment with heavy metals, such as cadium and arsenite, strongly induced expression of all the *Hsp90* genes (Milioni and Hatzopoulos, 1997). Two genes, *Hsc80* and *Hsp82* encode the yeast Hsp90. In studies of the yeast cognate gene *Hsc82* (*Hsc90*) it has been shown that while the amino acid sequence shares 97% identity with the Hsp82 protein, its constitutive expression is higher than its Hsp counterpart. However, *Hsc82* expression increases only slightly compared to *Hsp82* during heat shock (Borkovich *et al.*, 1989). Inactivation of either of the genes does not result in phenotypic changes at normal growth temperatures, but

inactivation of both genes is lethal (Borkovich *et al.*, 1989). In *Drosophila*, a single *Hsp90* (*Hsp80*) gene is responsible for both heat shock and developmental regulation (Blackman and Meselson, 1986). Heterozygous mutants display developmental abnormalities, while the complete absence of *Hsp80* is lethal (van der Straten *et al.*, 1997; Rutherford and Lindquist, 1998).

Southern blot analysis of barley genomic has revealed that the gene encoding the HvGrp94 transcript belongs to a family of closely related genes (Walther-Larsen *et al.*, 1993). Northern blot analysis has revealed that the HvGrp94 transcript is present at relatively constant levels in untreated barley leaves as well as other organs of the plant, however, a rapid accumulation of the transcript in response to heat shock in seedlings has been observed and it has also been seen to be induced upon infection with powdery mildew in barley leaves (Walther-Larsen *et al.*, 1993). In *Catharanthus roseus* (Madagascar periwinkle), Southern blot analysis revealed the presence of Grp94 as a single copy with closely related homologues indicating that it may be part of a family of closely related genes (Schroeder *et al.*, 1993). The protein is undetectable in plant extracts, but was found to be present at high levels in *C. roseus* cultures. Similar results were obtained with maize. Expression of this protein is also unaffected by various stress conditions, such as heat and it has been proposed that this protein has a chaperone role in the assembly and secretion of proteins in cells with a high rate of growth and division (Schroeder *et al.*, 1993).

These results indicate that while a gene may be expressed under certain conditions, it could belong to a group of related genes with differential regulation and function. In order to determine whether a gene of interest has more than one copy or belongs to a family of related genes the genomic DNA of the organism is probed with the gene. This is a simple method for detecting copy number as well as related genes and/or homologues. This chapter describes the isolation and sequence analysis of the full length *XVGrp94* cDNA from *X. viscosa*. The copy number of the gene was also determined using the full-length cDNA as a probe.

2.2. MATERIALS AND METHODS

2.2.1. Plant material

X. viscosa Baker plants were collected from the Cathedral Peak Nature Reserve located in the Drakensberg mountain range (Kwazulu Natal, South Africa), and were grown under glass house conditions as described by Sherwin and Farrant (1996).

2.2.2. Plant treatment

X. viscosa plants were dehydrated by withholding water under glass house conditions. Relative water content (RWC) was determined according to the formula in Appendix B (B.16.1).

2.2.3. Sequence Analysis

Isolation and sequence analysis of full length XVGrp94 cDNA clone

All non-proprietary solutions used for the RNA manipulations were treated with 0.01% diethylpyruvocarbonate (DEPC; B.14.4) and all Eppendorf tubes and pipette tips were autoclaved twice. Total RNA was isolated from *X. viscosa* leaves with a RWC of 30% using Trizol Reagent (Life Technologies, USA) according to the manufacturer's specifications. The RNA samples were quantified spectrophotometrically at a wavelength of 260 nm. Full-length cDNA transcripts were obtained using the SMART™ RACE cDNA Amplification Kit (Clontech, Palo Alto) according to the manufacturer's specifications. Polymerase chain reaction (PCR) was conducted on the cDNA using the Universal Primer (C.1.1) from the SMART™ RACE cDNA Amplification Kit and a gene specific reverse primer, Hsp90Rev3`A (C.1.2) according to specifications of the SMART™ RACE Kit.

The PCR products were electrophoresed on a 0.8% TBE gel (A.1.2.1) and a 2.6 kb band was gel purified (A.1.6.1) and cloned into the pGEM-T Easy vector (Promega, Madison, Wisconsin) according to the manufacturer's protocol. The ligation mix was used to transform *Escherichia coli* JM109 cells using the protocol described in Appendix A (A.1.5.1) and the cells were plated on LA supplemented with 100 mg/ml ampicillin (B.14.2), 0.5 mM IPTG (B.14.8) and 80 µg/ml X-Gal (B.14.19). White colonies were selected and screened by colony PCR (A.1.6.4.3) using the M13 Forward (C.1.7) and Reverse primers (C.1.8). The following protocol was used: (1) 94°C for 5 minutes (2) 94°C for 30 seconds (3) 55°C for 30 seconds (4) 72°C for 3 minutes (5) 72°C for 7 minutes. Steps 2-4 were repeated 25 times. The PCR products were electrophoresed on a 0.8% TBE gel. Recombinant plasmids containing the *XVGrp94* insert were harvested using the High Pure plasmid mini-preparation kit (Roche, Germany) according the manufacturer's recommendations and the DNA concentrations were determined (A.1.3.1).

The nucleotide sequence of *XVGrp94* cDNA clone was determined on both strands using the MegaBACE 500 (Molecular Dynamics, USA). The sequencing reactions were carried out using the DYEnamic ET Dye terminator sequencing kit (Molecular Dynamics, USA). All reactions were performed according to the manufacturer's instructions and cycle sequenced

on a GeneAmp PCR System 9700, Perkin Elmer (Applied Biosystems). The insert was sequenced using the following primers: M13 Forward primer (C.1.7), M13 Reverse primer (C.1.8), Hsp90atg (C.1.3), HomHspF (C.1.15), Rev5'D (C.1.9), Rev5`C (C.1.10), Hsp90F (C.1.11), HspRev5`A (C.1.12), Hsp90_5`A (C.1.13), and Hsp90Rev3`A (C.1.2). The sequence obtained was used to search for similarities in protein sequence databases using the BLAST network service (Altschul *et al.*, 1990). Amino acid comparisons and multiple sequence alignments were conducted using DNAMAN v4.0 (Lynnon Biosoft, 1997).

2.2.4. Southern Blot analysis

X. viscosa genomic DNA was extracted (A.1.1.1), quantified (A.1.3.1) and 10 μg DNA was digested in a 50 μl volume (A.1.6.5.1). Restriction enzyme digestions were conducted with the appropriate buffers and incubated at 37°C overnight. The digested genomic DNA was electrophoresed, at 20 V overnight, on a 0.8% TBE gel and transferred (A.1.4.1) to Hybond XL nylon membrane (Amersham, UK). To prepare the probe, the 2.6-kb cDNA was PCR-amplified (A.1.6.4.1) from the pGEM-T Easy vector using the Hsp90atg forward primer (C.1.3) and Hsp90Rev3`A reverse primer (C.1.14). The PCR reaction was performed using the following protocol: (1) 94°C for 3 minutes (2) 94°C for 30 seconds (3) 56°C for 30 seconds (4) 72°C for 3 minutes (5) 72°C for 7 minutes. Steps 2-4 were repeated 25 times. The resultant PCR product was electrophoresed on a 0.8% TBE gel and the 2.6-kb band excised and gel purified using a High Pure PCR purification kit (Roche, Germany) according to the manufacturer's specifications. The 2.6-kb *XVGrp94* DNA was labelled with [^{32}P]dCTP using the random primed labeling procedure (Roche, Germany) (A.1.6.2), purified (A.1.6.3) and used as a probe in the Southern hybridization reaction (A.1.4.2).

2.3. RESULTS AND DISCUSSION

2.3.1. Sequence analysis

The 1.5 kb cDNA originally identified (Ndima *et al.*, 2001), comprised an in complete open reading frame. To obtain a longer clone, the sequence of this 5`-truncated transcript was analysed and a reverse primer (Hsp90Rev3`A) was designed to be used in conjunction with the Universal Primer of the Smart™ Race Kit. The reverse primer was designed to amplify the region incorporating the seven nucleotides downstream of the TAA stop codon. The primer sequence itself incorporated these nucleotides as well as the sequence encoding the putative KDEL sequence immediately upstream of the stop codon (see Figure 2.4 below). The primer was also designed to be between 23 to 28 nucleotides in length and to have a GC content of 50-70% and a Tm of at least 65°C, as suggested by the manufacturers of the

SMART™ RACE cDNA amplification kit. A cDNA was isolated corresponding to the full-length *XVGrp94* transcript (Figure 2.1). The 2.6-kb band was isolated (Figure 2.1) and cloned into pGEMT-Easy, the recombinant clones were screened by PCR for the 2.6-kb insert (Figure 2.2) and the resulting construct was named pGEMT-XVGrp94 (C.2.1).

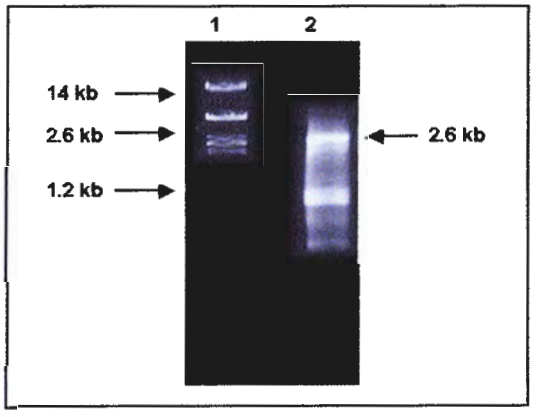

Figure 2.1: Product of the SMART™ RACE reaction. Lanes 1, λ *Pst*I DNA markers; 2, SMART-RACE product. Arrows on the left hand size indicate molecular sizes determined from the λ *Pst*I markers.

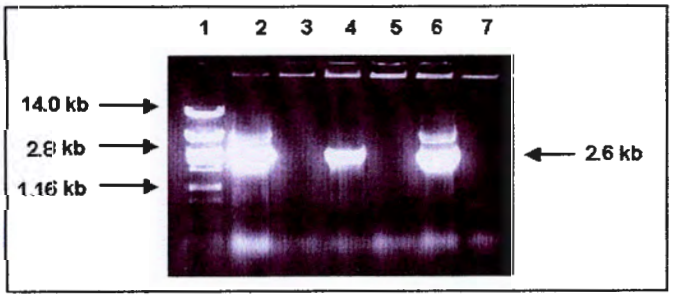

Figure 2.2: Products of the PCR-screening for pGEMT-Easy containing the full-length *XVGrp94* cDNA. Lanes 1, λ *Pst*I DNA markers; 2 to 6, PCR products from putative transformants; 7, negative control. Arrows on the left hand size indicate molecular sizes determined from the λ *Pst*I markers.

The cDNA was sequenced using a primer walking method in which sequence already obtained was used for the design of primers to sequence the next portion in the 3` direction (Figure 2.3). In some cases complementary primers were designed to obtain sequence in the 5`-direction. The 2.6-kb cDNA was truncated at the 3` end as the sequence ended 7 nucleotides after the stop codon and no longer included the poly-A tail. This sequence together with that already obtained for the 5`-truncated cDNA was assembled to give the nucleotide sequence shown in Figure 2.4. The 3059 bp full-length sequence contains a single large open reading frame (ORF) of 2.45 kb extending from the first ATG at position 114 to a translational stop (TAA) at position 2549, thus potentially encoding a polypeptide of 812 amino acid residues with a calculated molecular mass of 92.83 kDa. There is a consensus eukaryotic polyadenylation motif (AAUAAA) in the 3` region (nucleotides 3024-3029).

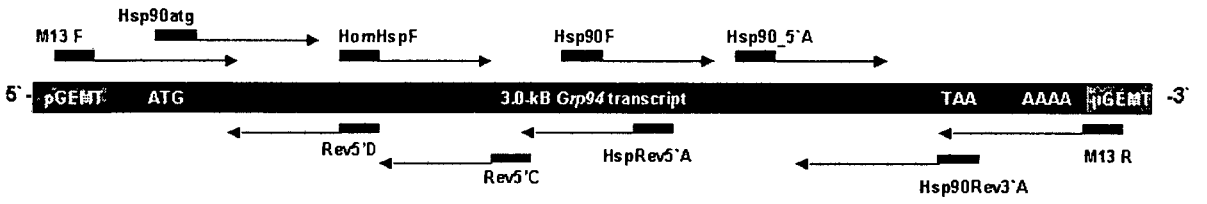

Figure 2.3: Sequencing of the *XVGrp94* cDNA. A section of the clone was sequenced, beginning with the M13 primers with recognition sites within the vector. The resulting sequence was used to design the next sets of primers. This was achieved for sequence in both the 5` to 3`and the 3` to 5` direction. Sequence was obtained in both directions and overlaps confirmed the correct alignment of contiguous portions of the sequence.

The deduced amino acid sequence was used for a computer-aided search for homologues in the databases. This search revealed that the cDNA sequence potentially encodes a new member of the Hsp90 family sharing a high degree of identity with Hsp90 from both plants and animals including the ER-resident GRP94s from vertebrates. The highest identities were to *Oryza sativa*, *Catharanthus roseus*, *Hordeum vulgare* and *Arabidopsis thaliana*, in the order of 82.74%, 81.97%, 80.10% and 79.90% respectively, with an overall identity of 85.5% (Figure 2.5).

The putative *X. viscosa* XVgrp94 sequence (Figures 2.4 and 2.5) contains the Hsp90 protein family signature Asn-Lys-Asp-Ile-Phe-Leu (NKDIFL, amino acids 104 to 109; Bairoch, 1992). It also contains two motifs characteristic of ER-located Hsp90s and these are (1) a region containing hydrophobic residues at the N-terminal end (amino acids 1-22), as determined by a hydrophobicity plot (Figure 2.6), which is a putative eukaryotic secretory signal sequence (Bairoch, 1992) and (2) the ER retention signal Lys-Asp-Glu-Leu (KDEL, amino acids 847 to 850) at the C-terminal end (Sorger and Pelham, 1987; Denecke *et al.*, 1991; Denecke *et al.*, 1992). On the basis of these similarities it is likely that the protein encoded by the *X.viscosa* cDNA described here represents an ER-resident homologue of the Grp94 found in vertebrate cells. This should be confirmed by localisation studies in which an antibody raised against the protein can be used to determine the subcellular location of the protein.

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1       CTAATACGACTCACTATAGGGCAAGCAGTGGTAACAACGCAGAGTACGCGGGAGCAGGTG

61      GTAACAACGTCAGAGTACGCGGGCAGCTCGCTACTTGAAGAACTCTCTCGAAGATGAGGA
1                                                          M  R

121     ATTGGTCGATCCCTCCTGCGCTCGTCCTCCTACTTCTGATTTCACTCTCCGCAATCCCTG
3       N  W  S  I  P  P  A  L  V  L  L  L  L  I  S  L  S  A  I  P

181     ATGGAGGCCGTAAGCTGCACGCGAACGCCGAGGAGAGCCGCGATGCCGACGAGCTTGTGG
23      D  G  G  R  K  L  H  A  N  A  E  E  S  R  D  A  D  E  L  V

241     ATCCGCCGAAGGTAGAGGAGAAGATCGCCGGCGTTCATGGAGGTCTTTCTACAGACGCCG
43      D  P  P  K  V  E  E  K  I  A  G  V  H  G  G  L  S  T  D  A

301     ATGTTGCCAAGAGGGAGGCGGAGTCGATGTCTAGGAAGAATTTGAGGAGCAATGCGGAGA
63      D  V  A  K  R  E  A  E  S  M  S  R  K  N  L  R  S  N  A  E

361     AATTCGAGTTTCAAGCTGAGGTTTCACGGCTAATGGATATCATCATCAACTCGCTCTACA
83      K  F  E  F  Q  A  E  V  S  R  L  M  D  I  I  I  N  S  L  Y

421     GTAACAAGGATATCTTCCTTAGAGAGCTCATCTCCAATGCGTCTGATGCTTTGGATAAGA
103     S  N  K  D  I  F  L  R  E  L  I  S  N  A  S  D  A  L  D  K

481     TAAGGTTCCTTTCCCTTACTGATAAGGAGGTATTGGGCGAGGGCGACAACACGAAGCTTG
123     I  R  F  L  S  L  T  D  K  E  V  L  G  E  G  D  N  T  K  L

541     AGATCATGATCAAGTTGGATAAGGAGAAGAAGATCCTCTCTATTAGAGATAGGGGTATTG
143     E  I  M  I  K  L  D  K  E  K  K  I  L  S  I  R  D  R  G  I

601     GTATGACCAAAGAGGACCTTATCAAGAATTTGGGTACTATTGCAAAGTCAGGAACCTCAG
163     G  M  T  K  E  D  L  I  K  N  L  G  T  I  A  K  S  G  T  S

661     CTTTTGTTGAGAAGATGCAGACAGGAGGTGATCTGAACCTCATCGGGCAGTTTGGAGTGG
183     A  F  V  E  K  M  Q  T  G  G  D  L  N  L  I  G  Q  F  G  V

721     GTTTCTACTCAGTATATCTAGTTTCTGACTATGTTGAAGTCATTAGCAAGCACAATGATG
203     G  F  Y  S  V  Y  L  V  S  D  Y  V  E  V  I  S  K  H  N  D

781     ACAAACAGTATGTTTGGGAATCTAAGGCTGATGGTGCATTTGCCATCTCCGAGGATACCT
223     D  K  Q  Y  V  W  E  S  K  A  D  G  A  F  A  I  S  E  D  T

841     GGAATGAGCCCTTGGGACGTGGAACTGAGATCCGTTTGCATCTGAGGGATGAAGCGAAGG
243     W  N  E  P  L  G  R  G  T  E  I  R  L  H  L  R  D  E  A  K

901     AGTACCTGGATGAGTCAAAATTGAAGGAGTTGGTTAAGAAGTATTCTGAATTTATCAACT
363     E  Y  L  D  E  S  K  L  K  E  L  V  K  K  Y  S  E  F  I  N

961     TCCCCATATACCTCTGGGCAAGCAAGGAGGTTGATGTTGAAGTTCCATCTGATGAAGAAG
283     F  P  I  Y  L  W  A  S  K  E  V  D  V  E  V  P  S  D  E  E

1021    AATCCAGTGATGTGGAGGAGAAATCTGAGAGTGAATCTTCTGAAGAAGAAATAGAAGAGG
303     E  S  S  D  V  E  E  K  S  E  S  E  S  S  E  E  E  I  E  E

1081    ACGATGCTGAGAAAAAGCCCAAAACAAAGACTGTCAAGGAAACAACCTATGAGTGGGAGC
323     D  D  A  E  K  K  P  K  T  K  T  V  K  E  T  T  Y  E  W  E

1141    TTTTAAATGATGTTAAGGCCATATGGCTTCGCAGCCCTAAAGAAGTTACTGATGAGGAAT
343     L  L  N  D  V  K  A  I  W  L  R  S  P  K  E  V  T  D  E  E

1201    ATACTAAGTTCTACCACTCTCTTGCTAAGGATTTTAGTGATGAAAAACCTTTAGCTTGGA
363     Y  T  K  F  Y  H  S  L  A  K  D  F  S  D  E  K  P  L  A  W

1261    GTCATTTTTCTGCTGAAGGTGATGTGGAATTCAAGGCTGTGCTCTTTGTGCCTCCTAAGG
383     S  H  F  S  A  E  G  D  V  E  F  K  A  V  L  F  V  P  P  K

1321    CACCACATGATCTGTACGAAAGTTACTACAATTCTCGTAAATCCAACTTGAAGCTATATG
403     A  P  H  D  L  Y  E  S  Y  Y  N  S  R  K  S  N  L  K  L  Y

1381    TCAGGCGTGTCTTCATTTCGGATGAATTTGACGAACTTCTCCCCAAGTACTTGAGCTTTT
423     V  R  R  V  F  I  S  D  E  F  D  E  L  L  P  K  Y  L  S  F

1441    TGATGGGTCTCGTTGACTCGGATACACTTCCACTGAATGTATCAAGAGAGATGCTTCAAC
443     L  M  G  L  V  D  S  D  T  L  P  L  N  V  S  R  E  M  L  Q
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1501    AGCATAGCAGCCTGAAGACAATTAAGAAGAAATTGATACGTAAAGCTCTTGATATGATTA
463     Q  H  S  S  L  K  T  I  K  K  K  L  I  R  K  A  L  D  M  I

1561    GAAAAATTGCTGATGAAGATCCTGATGAGTCTGACAAGGACCATTCGGAAGAAGCAGGTG
483     R  K  I  A  D  E  D  P  D  E  S  D  K  D  H  S  E  E  A  G

1621    AAGAGAATGAGAAGAAGGGATTGTACACTAAATTCTGGAATGAGTTTGGAAAATCAATAA
503     E  E  N  E  K  K  G  L  Y  T  K  F  W  N  E  F  G  K  S  I

1681    AGCTTGGCATTATTGAGGATGCACAAAATAGAAATCGCCTGGCCAAACTTCTGAGATTTG
523     K  L  G  I  I  E  D  A  Q  N  R  N  R  L  A  K  L  L  R  F

1741    AAACCACCAAGTCTGATGGTAAGCTTACATCGTTGGATAAGTACATATCTAGAATGAAAC
543     E  T  T  K  S  D  G  K  L  T  S  L  D  K  Y  I  S  R  M  K

1801    CTGGGCAGAAGGACATTTTCTACTTAACTGGAACCAGCAAAGAGCAGCTAGAGAAATCTC
563     P  G  Q  K  D  I  F  Y  L  T  G  T  S  K  E  Q  L  E  K  S

1861    CATTCCTTGAGGGGCTTAAGAAGAAGGACTATGAGGTCATTTTCTTCACTGACCCAGTTG
583     P  F  L  E  G  L  K  K  K  D  Y  E  V  I  F  F  T  D  P  V

1921    ATGAGTATTTGATGCAATACTTGATGGACTATGAGGACAAGAAATTCCAGAACGTGTCCA
603     D  E  Y  L  M  Q  Y  L  M  D  Y  E  D  K  K  F  Q  N  V  S

1981    AGGAGGGTCTCAAAATCGGGAAGGAGTCAAAGATCAAGGATCTCAAGGAGTCCTTCAAGG
623     K  E  G  L  K  I  G  K  E  S  K  I  K  D  L  K  E  S  F  K

2041    AGCTCACCAGTTGGTGGAAAGAAGCTCTTTCCAGTGAGAATGTGGACTCCGTGAAGATTA
643     E  L  T  S  W  W  K  E  A  L  S  S  E  N  V  D  S  V  K  I

2101    GCAACCGGTTGGATAACACTCCTTGCGTTGTTGTCACATCGAAATATGGTTGGAGTGCAA
663     S  N  R  L  D  N  T  P  C  V  V  V  T  S  K  Y  G  W  S  A

2161    ACATGGAGAAGATTATGCAGTCTCAGACTCTTTCCGATGCCAGCAAGCAGGCATACATGC
683     N  M  E  K  I  M  Q  S  Q  T  L  S  D  A  S  K  Q  A  Y  M

2221    GCGGGAAGAGGGTGCTCGAGATTAACCCAAGACACCCTATCATTAAAGAACTTCGTGAAA
703     R  G  K  R  V  L  E  I  N  P  R  H  P  I  I  K  E  L  R  E

2281    GAGTTGCCGTGGACCCTCAGGATGAAAACATCAAGCAAACAGCGAAGCTGATTTACCAAA
723     R  V  A  V  D  P  Q  D  E  N  I  K  Q  T  A  K  L  I  Y  Q

2341    CAGCTCTCATGGAGAGTGGATTCCTGATGAATGATCCGAAAGAGTTCGCAACAAGTATAT
743     T  A  L  M  E  S  G  F  L  M  N  D  P  K  E  F  A  T  S  I

2401    ACAGTTCTGTTAAATCCAGCTTGAATATTAGTCCAGATGCCAAAGTCGAAGAGGAGGAGG
763     Y  S  S  V  K  S  S  L  N  I  S  P  D  A  K  V  E  E  E  E

2461    ATACTGATGAGCCGGAGGTGGAAGAAAAGGAATCTGCTTCAAGCAAGGGATCAGAAGAGG
783     D  T  D  E  P  E  V  E  E  K  E  S  A  S  S  K  G  S  E  E

2521    CAGAGGATTTCAGCGCCAAGGATGAGTTGTAACTCACGCTAAGTTAACATCTTTTTTGTT
803     A  E  D  F  S  A  K  D  E  L  *

2581    TCGGATAATGTGGTTAGGAGCAGCCGTGCCACTGAGGCTGAATTATGCTTTAAGTGAGAA
2641    AAACGAGCCAAATGGCTCTGACGATTTTACTAGCTGAATTTTGCTGTTCTTTAACGAGTA
2701    GGATTTTGGTTTTCAATTCTACCGTGTGCTCTTAATTAATCCACCTTCATGTTGTTGTGA
2761    CATGTTCCTTATTTTCTAATGTGAGTGTGAATTACAGTCTTAGGATTGTATCATTGATCA
2821    ACGGTGAGATTCCGGAAGGGTATAACTGATGAACTAGCGAGATTATGGGTGGTTGCTGAG
2881    TAGGTGTTCTCCAGCAATTTTTCAGCAAGTTTCGATGCGCTGTGACTGCTTTATTTTCTA
2941    TTTCTAGAAGTTTTTTAAATAATGTTGGGATTTTTCCCTATGCTTTGTGAGTATCGAGCA
3001    ACTAAAACTATCTTGATAGCGTTAATAAATACTGTTACATTTTAAAAAAAAAAAAAAAAA
```

Figure 2.4: Nucleotide and deduced amino acid sequence of *XVGrp94*. The putative start and stop codons are represented in bold. The nucleotide sequence of the primers used in the SMART™ RACE reaction are underlined. The eukaryotic secretory signal sequences, the conserved catalytic glutamate (Glu-33), the highly conserved *Hsp90* family signature, NKDIFL and the ER retention signal, KDEL are all underlined.

```
XVGrp94          MRNWSIPPALVLLLLISLSAIPDGGRKLHANAEESRDADELVDPPKVEEKIAGVHGGLST 60
Oryza            MRKWALSSALLL-LLLTTL--PDPAKKLQVNADDST--DELVDPPKVEEKIGGVPHGLST 55
Catharanthus     --MRKWTVPSVLFLLCPSLSSSCQGRKIHANAEADSDAP--VDPPKVEDKIGAVPNGLST 56
Hordeum          MRKWALSCALLLVLLLTTL--PDPAKKLQVNAEESS--DEVGDFPKVEEKLGAVPHGLST 56
Arabidopsis      --MRKRTLVSVLFLFSLLFLLPDQGRKLHANAEESSDDVTDPPKVEEKIGGHG---GLST 55
                           .* *           .*.. **.                    ****

XVGrp94          DADVAKREAESMSRKNLRSNAEKFEFQAEVSRLMDIIINSLYSNKDIFLRELISNASDAL 120
Oryza            DSEVVQREAESISRKTLRSSAEKFEFQAEVSRLMDIIINSLYSNKDIFLRELISNASDAL 115
Catharanthus     DSDVAKREAESMSMRNLRSDAEKFEFQAEVSRLMDIIINSLYSNKDIFLRELISNASDAL 116
Hordeum          DSEVVQRESESISRKTLRNSAEKFEFQAEVSRLMDIIINSLYSNKDIFLRELISNASDAL 116
Arabidopsis      DSDVVHRESESMSKKTLRSNAEKFEFQAEVSRLMDIIINSLYSNKDIFLRELISNASDAL 115
                 *..* .**.**.* ..**  ****************************************

XVGrp94          DKIRFLSLTDKEVLGEGDNTKLEIMIKLDKEKKILSIRDRGIGMTKEDLIKNLGTIAKSG 180
Oryza            DKIRFLALTDKEVLGEGDTAKLEIQIKLDKEKKILSIRDRGIGMTKEDLIKNLGTIAKSG 175
Catharanthus     DKIRFLALTDKEILGEGDTAKLEIQIKLDKEKKILSIRDRGIGMTKEDLIKNLGTIAKSG 176
Hordeum          DKIRFLALTDKEVMGEGDTAKLEIQIKLDKENKILSIRDRGVGMTKEDLIKNLGTIAKSG 176
Arabidopsis      DKIRFLALTDKDVLGEGDTAKLEIQIKLDKAKKILSIRDRGIGMTKEDLIKNLGTIAKSG 175
                 ******.****...****..**** *****  *********.******************

XVGrp94          TSAFVEKMQTGGDLNLIGQFGVGFYSVYLVSDYVEVISKHNDDKQYVWESKADGAFAISE 240
Oryza            TSAFVEKVQTGGDLNLIGQFGVGFYSVYLVADYVEVISKHNDDKQHVWESKADGSFAISE 235
Catharanthus     TSAFVEKMQTSGDLNLIGQFGVGFYSVYLVPDYVEVISKHNDDKQYIWESKADGAFAISE 236
Hordeum          TSAFVEKMQTGGDLNLIGQFGVGFYSVYLVADYVEVVSKHNDDKQYVWESKADGSFAISE 236
Arabidopsis      TSAFVEKMQSSGDLNLIGQFGVGFYSAYLVADYIEVISKHNDDSQYVWESKANGKFAVSE 235
                 *******.*. *************** *** **.**.****** * .***** * **.**

XVGrp94          DTWNEPLGRGTEIRLHLRDEAKEYLDESKLKELVKKYSEFINFPIYLWASKEVDVEVPSD 300
Oryza            DTWNEPLGRGTEIRLHLRDEAKEYVEEDKLKDLVKKYSEFINFPIYLWATKEVDVEVPAD 295
Catharanthus     DVWNEPLGRGTEIRLHLRDEAQEYLDEFKLKELVKRYSEFINFPIYLWASKEVEVEVPAE 296
Hordeum          DTWNEPLGRGTEIKLHLRDEAKEYLEEGKLKDLVKKYSEFINFPIYLWATKEVDVEVPAD 296
Arabidopsis      DTWNEPLGRGTEIRLHLRDEAGEYLEESKLKELVKRYSEFINFPISLWASKEVETEVPVE 295
                 * ***********.******* **..* ***.***.********* ***.***. *** .

XVGrp94          EEESSDV--EE-KSESESSEEEIEEDDA----EKKPKTKTVKETTYEWELLNDVKAIWLR 353
Oryza            EDESSESSEEE-ESSPESTEEEETEEGE----EKKPKTKTVKETTTEWELLNDVKAIWLR 350
Catharanthus     EDDSSDD--EDNKSESSSSEEGEEEETEKEEDEKKPKTKKVKETTYEWELLNDMKAIWLR 354
Hordeum          EE---ESNEEE-ESTTETTEEEETEDDE----EKKPKTKTVKETTTEWELLNDMKAVWLR 348
Arabidopsis      EDESADEETETTST-----EEEKEEDAEEEDGEKKQKTKKVKETVYEWELLNDVKAIWLR 350
                 *.   .   *   .     **   *.      *** *** ****  *******.**.***

XVGrp94          SPKEVTDEEYTKFYHSLAKDFSDEKPLAWSHFSAEGDVEFKAVLFVPPKAPHDLYESYYN 413
Oryza            SPKEVTEEEYTKFYHSLAKDFGDDKPLSWSHFTAEGDVEFKALLFVPPKAPHDLYESYYN 410
Catharanthus     NPKDVTDDEYTKFYHSLAKDFSEEKPLAWSHFTAEGDVEFKAFTLLPPKAPQDLYESYYN 414
Hordeum          SPKEVTEEEYAKFYHSLAKDFGDDKPMSWSHFSAEGDVEFKALLFVPPKAPHDLYESYYN 408
Arabidopsis      SPKEVTEEEYTKFYHSLSKDFTDEKPMAWSHFNAEGDVEFKAVLYVPPKAPHDLYESYYN 410
                  **.**..**.******.*** ..**..**** *********   .*****.********

XVGrp94          SRKSNLKLYVRRVFISDEFDELLPKYLSFLMGLVDSDTLPLNVSREMLQQHSSLKTIKKK 473
Oryza            SNKSNLKLYVRRVSISDEFDELLPKYLSFLKGLVDSDTLPLNVSREMLQQHSSLKTIKKK 470
Catharanthus     SNKSNLKLYVRRVFISDEFDELLPKYLNFLKGLVDSDTLPLNVSREMLQQHSSLKTIKKK 474
Hordeum          ANKSNLKLYVRRVFISDEFDDLLPKYLSFLMGIVDSDTLPLNVSREMLQQHSSLKTIKKK 468
Arabidopsis      SNKANLKLYVRRVFISDEFDELLPKYLSFLKGLVDSDTLPLNVSREMLQQHSSLKTIKKK 470
                 . *.********** ******.****** ** *.**************************

XVGrp94          LIRKALDMIRKIADEDPDES---DKDHSEEAGEENEKKGLYTKFWNEFGKSIKLGIIEDA 530
Oryza            LIRKALDMIRKLAEEDPDEYSNKDKTD-EEKSAMEEKKGQYAKFWNEFGKSVKLGIIEDA 529
Catharanthus     LIRKALDMIRKIADEDPDEANDKDKKEVEESTDNDEKKGQYAKFWNEFGKSIKLGIIEDA 534
Hordeum          LIRKALDMIRKLAEEDPDEYSNKEKTD-DEKSAMEEKKGQYAKFWNEFGKSVKLGIIEDA 527
Arabidopsis      LIRKALDMIRKLAEEDPDEIHDDEKKDVEKSGENDEKKGQYTKFWNEFGKSVKLGIIEDA 530
                 ***********.*.*****    .*   .       **** *.*********.********

XVGrp94          QNRNRLAKLLRFETTKSDGKLTSLDKYISRMKPGQKDIFYLTGTSKEQLEKSPFLEGLKK 590
Oryza            TNRNRLAKLLRFESTKSEGKLASLDEYISRMKPGQKDIFYITGSSKEQLEKSPFLERLTK 589
Catharanthus     ANRNRLAKLLRFESTKSEGKLTSLDQYISRMKSGQKDIFYITGTSKEQLEKSPFLERLTK 594
Hordeum          TNRNRLAKLLRFESSKSDGKLVSLDEYISRMKSGQKDIFYLTGSSKEQLEKSPFLEQLTK 587
Arabidopsis      ANRNRLAKLLRFETTKSDGKLTSLDQYIKRMKKSQKDIFYITGSSKEQLEKSPFLERLIK 590
                  ************..**.*** *** ** ***  ******.**.************ * *
```

```
XVGrp94          KDYEVIFFTDPVDEYLMQYLMDYEDKKFQNVSKEGLKIGKESKIKDLKESFKELTSWWKE 650
Oryza            KNYEVIYFTDPVDEYLMQYLMDYEDKKFQNVSKEGLKLGKDSKLKDLKESFKELTDWWKK 649
Catharanthus     KNYEVILFTDPVDEYLMQYLMDYEDKKFQNVSKEGLKIGKDSKDKELKESFKELTKWWKG 654
Hordeum          KNYEVIYFTDPVDEYLMQYLMDYEDKKFQNVSKEGLKLGKDSKLKDLKESFKELTDWWKK 647
Arabidopsis      KGYEVIFFTDPVDEYLMQYLMDYEDKKFQNVSKEGLKVGKDSKDKELKEAFKELTKWWKG 650
                 * **** ******************************.**.** *.***.***** ***

XVGrp94          ALSSENVDSVKISNRLDNTPCVVVTSKYGWSANMEKIMQSQTLSDASKQAYMRGKRVLEI 710
Oryza            ALDTESVDSVKISNRLSDTPCVVVTSKYGWSANMEKIMQSQTLSDASKQAYMRGKRVLEI 709
Catharanthus     ALASENVDDVKISNRLANTPCVVVTSKYGWSSNMERIMQSQTLSDASKQAYMRGKRVLEI 714
Hordeum          ALDTEGIDSVKISNRLHNTPCVVVTSKYGWSSNMEKIMQAQTLSDASKQAYMRGKRVLEI 707
Arabidopsis      NLASENVDDVKISNRLADTPCVVVTSKFGWSANMERIMQSQTLSDANKQAYMRGKRVLEI 710
                  * .* .* *******  *********.***.***.***.****** *************

XVGrp94          NPRHPIIKELRERVAVDPQDENIKQTAKLIYQTALMESGFLMNDPKEFATSIYSSVKSSL 770
Oryza            NPRHPIIKELRDKVAQDSESESLKQTAKLVYQTALMESGFNLPDPKDFAFSIYRSVQKSL 769
Catharanthus     NPRHPIIKELRERVVKDAEDESVKQTARLMYQTALMESGFMLNDPKEFASSIYDSVKSSL 774
Hordeum          NPRHPIIKELRDKVAQDSDSEGLKQTARLVYQTALMESGFNLPDPKDFASSIYRSVQKSL 767
Arabidopsis      NPRHPIIKELKDRIASDPEDESVKETAQLMYQTALIESGFILTDPKDFAARIYNSVKSGL 770
                 **********....  *   * .*.**.*.*****.**** . ***.**  ** **.  *

XVGrp94          NISPDAKVEEEED----------TDEPEVEEKESASSKGSEE-AEDFSA-KDEL 812 100%
Oryza            DLSPDAAVEEEEE----------VEEAEVEEKE--SSNIK-EEAEPSSYDKDEL 810 82.74%
Catharanthus     KISPDATVEEEDD----------TEEAEAESGTTESSAAEDAGAETLDL-KDEL 817 81.97%
Hordeum          DLSPDAAVEEEEE----------VEEPEVEEKE--SAKQEAEEPEHEQYDKDEL 809 80.10%
Arabidopsis      NISPDAVADEEIEAAEEPETSEATETKSDDLAGGLNIEAEPVEQQEENT KDEL 823 77.90%
                  .****  .** .           .    .                .      .
```

Figure 2.5: Amino acid comparison of XVGrp94 with related proteins obtained from a computer search using the BLAST network service. An asterisk (*) indicates "identity" with the XVGrp94 sequence while a dot represents "similarity". Percentages following the sequences indicate the percentage identity to XVGrp94 as calculated by the multiple sequence alignment function of DNAman. The sequences are: Oryza, a Hsp90 from *O. sativa* (GenBank accession number: BAA90487); Catharanthus, a Grp94-related protein from *C. roseus* (Schroeder *et al.*, 1993; GenBank accession number: P35016); Hordeum, a Grp94-related protein from *H. vulgare* (Walther-Larsen *et al.*, 1993; GenBank accession number: P36183); Arabidopsis, an Hsp90-like protein from *A. thaliana* (Mayer *et al.*, 1999; GenBank accession number: NP_194150).

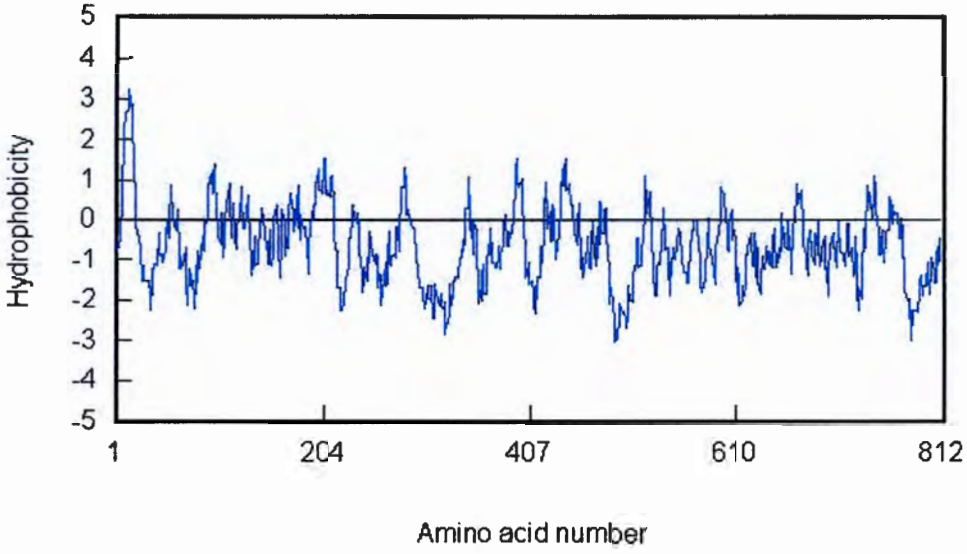


Figure 2.6: Hydrophobicity plot of XVGrp94.

The question as to whether Hsp90 binds and hydrolyses ATP has been a controversial one (Wiech *et al.,* 1992; Csermely *et al.,* 1993; Johnson and Toft, 1994; 1995; Jakob *et al.,* 1996; Grenert *et al.,* 1997; Scheibel *et al.,* 1997, 1998), with recent findings indicating that this does indeed occur (Grenert *et al.,* 1997; Prodromou *et al.,* 1997b; Scheibel *et al.,* 1997; Obermann *et al.,* 1998; Panaretou *et al.,* 1998; Scheibel *et al.,* 1998;Grenert *et al.,* 1999; Panaretou *et al.,* 1999; Prodromou *et al.,* 1999; Prodromou *et al.,* 2000). Hsp90 was classified as a member of a newly emerging ATPase family after an unexpected, yet remarkable, similarity in overall tertiary structure between the N-terminal domain of Hsp90 and the N-terminal ATP binding domain of the bacterial type II topoisomerase, DNA gyrase B of *E.coli* was observed (Wigley *et al.,* 1991). There is a high degree of similarity in the conformation of the bound ATP and many of the protein residues that interact with the bound ATP are conserved between the two proteins (Prodromou *et al.,* 1997b). Of particular interest is the conserved glutamate residue in the gyrase protein E42 (Glu-42), which has been identified as the catalytic center for ATP hydrolysis (Jackson and Maxwell, 1993). This corresponds to E33 in the yeast Hsp90 protein and E47 in the human protein and it is also conserved in the XVGrp94 protein at position E33. This indicates that XVGrp94, like the Hsp90 from yeast and humans, is equipped for ATPase activity (Prodromou *et al.,* 1997b). Other residues lying within the ATP-binding pocket are also conserved in XVGrp94. These include L77 (corresponds to L77 in the yeast protein), V133 (corresponds to V136 in the yeast protein), T175 (corresponds to T171 in the yeast protein) and L176 (corresponds to L173 in the yeast protein) (Stebbins *et al.*, 1997). Of particular interest is D95 (corresponding to D93 in yeast), as it is at the bottom of the ATP binding pocket in an otherwise mostly hydrophobic environment and it is conserved in all known Hsp90 homologues from 35 species (Stebbins *et al.*, 1997). The conservation of these residues indicate that XVGrp94 may have an ATP-binding pocket that is very similar to that of the Hsp90 protein from yeast and humans and that it also possibly possesses ATPase activity. Solving the tertiary structure of this protein will reveal more about ATP-binding and hydrolysis in this protein.

It must also be considered that this Hsp90 protein may reside within the ER. The tertiary structure has only been solved for the cytosolic member of this group of proteins and there may therefore be some structural differences. Grp94 has also been shown to be an ATP binding protein (Li and Srivastava, 1993; Csermely *et al.,* 1995; Rosser *et al.,* 2000) and ATP binding has been shown to lead to autophosphorylation of the protein as well as an ATPase activity (Li and Srivastava, 1993). The N-terminal domain of Grp94 is predicted to

have a tertiary structure similar to that of Hsp90 (Gerloff *et al.*, 1997). If this is the case it may contain an ATP binding site similar to that in Hsp90 (Csermely *et al.*, 1998).

2.3.2. Southern blot analysis

Figure 2.7A is a representation of the cDNA with the position of the restriction sites used in the digestion of the genomic DNA. To carry out Southern blot analysis of genomic *X. viscosa* DNA the cDNA sequence was used as a reference for choosing the appropriate restriction enzymes. It must therefore be borne in mind that the effect of the presence of introns in the chromosomal copy of the gene was not taken into account. *X. viscosa* genomic DNA was digested with each of the restriction enzymes *Hind*III, *Bam*HI, *Eco*RI, *Bgl*I or *Pst*I, and was analysed using the complete cDNA as a probe (Figure 2.7B and C).

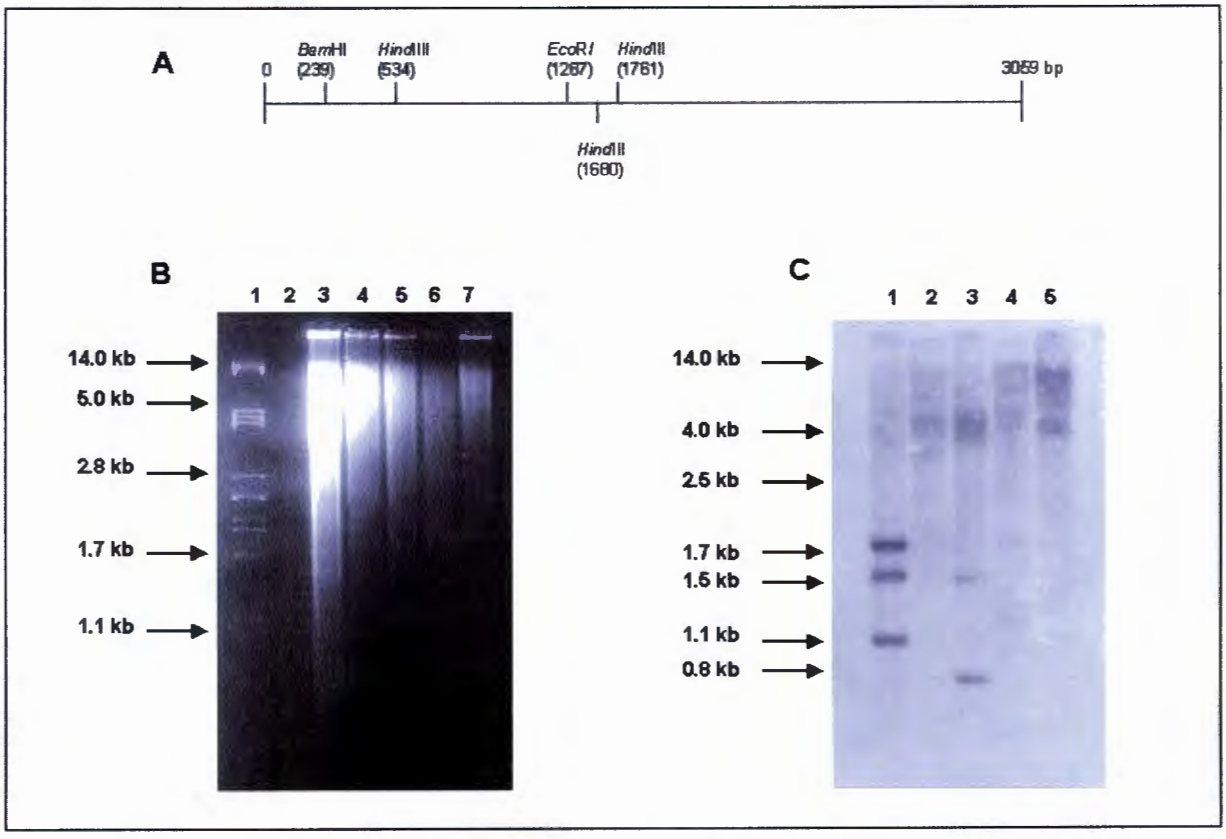


Figure 2.7: Southern blot of *X. viscosa* genomic DNA. A, Position of the restriction sites in the cDNA for the enzymes used in the digestion of the DNA for the genomic blots. B, Gel photograph of the digested genomic DNA. 10 µg of *Xerophyta viscosa* genomic DNA was digested with *Hind*III (lane 3), *Bam*HI (lane 4), *Eco*RI (lane 5), *Bgl*I (lane6) and *Pst*I (lane 7). Lane 1 is λ *Pst*I DNA marker and lane 2 has been left blank. Arrows on the left hand size indicate molecular sizes determined from the λ *Pst*I markers. C, Southern blot analysis of the gel. Lane 1, *Hind*III; 2, *Bam*HI; 3, *Eco*RI; 4, *Bgl*I, 5, *Pst*I. The full length *XVGrp94* cDNA was used as a probe. The arrows indicate the size of the bands detected as determined from the λ *Pst*I DNA markers.

According to the cDNA sequence the *Hind*III digest should result in 1.1-kb band an 81-bp band. The 1.1-kb band is present and the 81-bp band was, presumably, too small to detect. There are, however, two other bands observed (1.7 kb and 1.5 kb) and it is thought that these could be due to the presence of *Hind*III sites flanking the *XVGrp94* gene within the genome. *Bam*HI only cuts once within the cDNA and there is some evidence of a 2.5-kb band indicating that the enzyme did cut once within the gene. *Eco*RI also only cuts once within the cDNA, but two bands of 1.5 kb and 0.8 kb were observed. The 1.5-kb band could arise as a result of an additional *Eco*RI site flanking the gene, but the 0.8-kb band could be explained by the presence of an intron with an additional *Eco*RI site. There are no recognition sites for *Bgl*II within the cDNA sequence, however two bands of 1.7 kb and 1.2 kb were detected. *Pst*I does not cut within the cDNA and one band of 4.0 kb was detected, indicating that the chromosomal copy of the gene is not cut by *Pst*I.

It was observed that the banding pattern obtained from digestion of chromosomal DNA was different to that predicted by the cDNA sequence, even taking into account the possibility of flanking recognition sites. This could be due to either the presence of introns within the chromosomal copy of the gene or the presence of a closely related homologue with a different restriction pattern. In order to determine the reason for the results observed, the chromosomal copy/copies of the gene should be isolated. Sequence information and restriction endonuclease analysis of this will reveal which enzymes cut within the gene, thereby making it easier to determine copy number from the Southern blot. The Southern blot shows that *Pst*I does not cut within the chromosomal copy of the *XVGrp94* gene and therefore digestion of genomic DNA with this enzyme, and construction of a library, should result in the isolation of a full-length chromosomal copy of *XVGrp94*. It is known that there are ER homologues and cytosolic homologues, and as mentioned above (see section 2.1) many of the plant species studied were found to contain multiple copies of the *XVGrp94* gene, except in the case of *C. roseus*. Possibly lower stringency washes or longer exposure times may reveal the presence of less identical homologues. This Southern blot was only left to expose for three days and longer exposures of up to 10 days may reveal other closely related genes.

2.4. CONCLUSION

Sequence analysis confirmed the identity of the cDNA clone as XVGrp94 and Southern blot analysis showed the presence of the gene within the *X. viscosa* genome. Analysis of expression of this protein under different environmental stresses would serve two purposes. Firstly, to validate the approach by which this clone was isolated and confirm that it is indeed induced by conditions of drought stress. Secondly, it would provide information regarding other stress responses that this protein could be involved in. The results of this analysis are presented in the next chapter.

CHAPTER THREE

ANALYSIS OF EXPRESSION PATTERNS OF *XVGrp94*

SUMMARY

The *XVGrp94* transcript size was determined by northern blot analysis. Total RNA was extracted from leaves of a hydrated *X. viscosa* plant and probed with a 1-kb C-terminal region of the *XVGrp94* cDNA. A 2.8-kb transcript was detected. Northern and western blot analyses were conducted in order to investigate the expression pattern of *XVGrp94* in response to the following environmental stresses: heat (42°C), dehydration, high light (1500 $\mu Mol.m^{-2}.s^{-1}$), cold (4°C), high salinity (150 mM). The response to exogenous ABA (100 μM) was also studied. Total RNA was extracted from leaf samples of a heat-treated *X. viscosa* plant as well as from leaf samples of a plant subjected to dehydration. The RNA was probed with a 1-kb C-terminal region of the *XVGrp94* cDNA. The results of the northern analyses were negative and the reasons for this were not established. Total protein was extracted from leaves of *X. viscosa* plants subjected to all of the treatments described above and probed with an antibody raised against Grp94 from *C. roseus* (Madagascar periwinkle). XVGrp94 expression increased dramatically in response to heat stress and dehydration. There was a slight increase in response to high salinity and very little change in expression in response to exogenous ABA. No change in XVGrp94 expression was observed in response to high light or cold stress.

3.1. INTRODUCTION

3.1.1. *Hsp90* expression in plants

In maize (Marrs *et al.*, 1993) and tomato (Koning *et al.*, 1992) the expression of the *Hsp90* gene family members have been shown to be developmentally regulated. An *Hsp90* transcript was shown to be strongly expressed in maize during pre-meiotic and meiotic prophase stages of pollen development and in embryos (Marrs *et al.*, 1993). In tomato an *Hsp90* (*Hsc80*; heat shock cognate 80) mRNA is seen to be abundant in shoot and root apices and in fertilized ovaries (Koning *et al.*, 1992). *Hsp90* (*Hsp83*) homologues in *P. nil* are regulated by both heat shock and light. Seedlings of this species can be induced to flower by a dark period of sufficient length (10 hours). The expression of *Hsp83B* was up-regulated by the photoperiod and was located in the cotyledons of the seedling. The

cotyledon is the primary sensor that signals to the apical meristem to change from vegetative growth to reproductive growth and it would therefore appear that Hsp83 has a role in this signaling process. *Hsp83A* was found to be the most heat-inducible *Hsp83* gene in cotyledons, whereas the others exhibited a low level of heat induction (Felsheim and Das, 1992). *Hsp90* has been found in all tissues of *Brassica napus*, but transcript levels in the different tissues vary (Krishna *et al.,* 1995). Again Hsp90 appears to have a role in growth and development of the plant as high levels of *Hsp90* mRNA and protein were found in young and rapidly dividing tissues such as shoot apices and flower buds. A significant increase in *Hsp90* mRNA levels has also been detected in seedlings exposed to 5°C and similar cold-regulation of *Hsp90* mRNA in spinach (*Spinacea oleracea*) has also been confirmed. These results suggest a role for Hsp90 in adaptation to low temperature stress. The expression of Hsp90 mRNA and protein was also found to increase in response to heat, but not to dehydration stress, supporting a protective role during temperature stress in *B. napus* (Krishna *et al.,* 1995).

The *Arabidopsis Hsp90* gene family comprises seven genes (Krishna and Gloor, 2001), four of which are cytosolic and the remaining three are organellar. The cytosolic *Hsp90*s are strongly induced by heat shock at 37°C and 42°C (Conner *et al.,* 1990; Milioni and Hatzopoulos, 1997). The organelle members, however, displayed different gene expression profiles. The *Hsp90* homologue, thought to reside within peroxisomes, was not expressed at elevated temperatures, whereas the other organelle homologue, which is thought to be directed to the chloroplasts, showed higher levels of mRNA accumulation at 37°C for 1 hour, while transcription almost ceased and mRNA accumulation was very low at 42°C. Treatment of *Arabidopsis* with cadium and arsenite strongly stimulated the accumulation of all the *Hsp90* mRNAs (Takahashi *et al.,* 1992; Milioni and Hartzopoulos, 1997).

To date the functional significance of these mRNA expression patterns is unclear. It is possible that Hsp90 plays an important role in regulating important cellular processes in a similar manner to that of its animal counterpart. This is supported by the apparent developmental regulation. It may have a role in regulating cell signaling and cell cycle progression in a similar way to that of Hsp90 in animal cells. The increase in expression of *Hsp90* in response to stress would indicate a possible chaperone function of this protein to protect proteins from damage during stress, as postulated for the mammalian counterpart.

3.1.2. *Grp94* expression in plants

While Hsp90 and Grp94 share a high degree of sequence similarity, their expression patterns differ. Initial studies showed that treatment of developing bean cotyledons with tunicamycin, an inhibitor of N-glycosylation, caused the accumulation of malfolded proteins within the ER, and enhanced the synthesis of Grp94 (D'Amico *et al.,* 1992), suggesting a chaperone function for this protein. Grp94 has also been found to increase in barley in response to heat shock and in response to attack by the causitive agent of powdery mildew, *Erysiphe graminis* (Walther-Larsen *et al.,* 1993). Barley is resistant to attack by this fungus and its resistance has been found to develop concomitantly with the formation of papillae, i.e. cell wall depositions that form below the fungal hyphae. The increased expression of *Grp94* might simply be a result of an enhanced requirement for the protein as the secretory protein traffic is increased in the ER during the formation of papillae. Papillae consist of callose, phenolic compounds and proteins and are formed at the inner side of the wall, outside the plasmalemma and their formation would therefore result in an increase in the ER transport process. It is also proposed Grp94 is involved in calcium ion regulation. In plant cells, changes in the calcium concentrations are believed to have a role in the signal transduction pathways, which result in the physiological changes observed in response to pathogen attack. The pathogen attack may disrupt ER calcium stores and Grp94 may act to restore the calcium balance. Alternatively, Grp94 may be involved in mobilization and regulation of calcium ions to facilitate ER protein transport during the stress (Walther-Larsen *et al.,* 1993).

Schroeder and co-workers (1993) showed constitutive expression of Grp94 in cell cultures of maize and madagascar periwinkle but not in the young plants of these species. Various types of stress, including high temperature, high sucrose concentration, pathogen elicitors or elicitor active substances have little or no effect on the expression of the protein in periwinkle. *Grp94* expression is, however, induced by heat in maize cell cultures. The observation that *Grp94* expression does not increase in response to high sucrose concentrations indicate that the periwinkle homologue, although being homologous a group of glucose regulated proteins, is in fact not itself regulated by glucose (Shroeder *et al.,* 1993). Suspension cultures have a high rate of cell growth and division, and this includes the extensive synthesis and secretion of cell wall components and a chaperone function for Grp94 has been proposed in the assembly and processing of cell wall components and other secreted proteins. This is in agreement with the results obtained from studies by Walther-Larsen *et al* (1993), where a similar explanation was offered for the pathogen induced expression of the ER located *Hsp90*.

In tobacco, a basal expression level of *Grp94* was detected indicating a role for this protein in routine protein biosynthesis in the ER. It was also discovered to be upregulated during seed germination (Denecke *et al.,* 1995). Alpha amylase constitutes 50% of the newly synthesised proteins in barley aleurone tissue. During seed germination, hydrolase secretion is needed for a relatively short time to mobilise starch reserves from the endosperm. It was found that induction of hydrolase, in barley aleurone cells was accompanied by a coordinated increase in the mRNA levels for *Grp94* (Denecke *et al.;* 1995), indicating a role for Grp94 in the secretion of proteins during this stage.

From all the examples presented above, this protein appears to have a chaperone function in plants, in some cases during stress, but also during development. This is to be expected as during development many proteins are being made and secreted. Under conditions of stress, damaged or misfolded proteins accumulate in the ER and must be folded and assembled or sent to be degraded. This is in addition to nascent proteins, which must be prepared quickly during stress to serve their functions. Also certain developmental stages require large amounts of proteins to be secreted and Grp94 may have a role in this process. Identifying substrate proteins will help to elucidate the exact function of this protein during various conditions and may explain why there is no universal expression pattern observed.

3.1.3. Aims

This chapter describes the analysis of *XVGrp94* expression in response to various environmental stresses. Since *XVGrp94* is thought to be a molecular chaperone, it is hypothesised that it may respond to certain environmental stresses, such as heat. Northern analysis was attempted to determine the profile of gene expression during the stresses. This, however, was not successful. Western blot analysis indicated that XVGrp94 may have a role during conditions of stress in *X. viscosa*.

3.2. MATERIALS AND METHODS

3.2.1. Plant material

Xerophyta viscosa Baker plants were collected from the Cathedral Peak Nature Reserve located in the Drakensberg mountain range (Kwazulu Natal, South Africa), and were grown under glass house conditions as described by Sherwin and Farrant (1996). Material was obtained from a Madagascar periwinkle (*C. roseus)* plant, collected from a residential garden in Cape Town, South Africa.

3.2.2. Plant treatments

X. viscosa plants were subjected to a number of environmental stresses described below. All treatments, unless specified, were conducted on whole plants in the phytotron. The conditions in the phytotron were constant with a light/dark cycle of 16 hours light and 8 hours dark, a temperature of 24°C and a light intensity of 380 μMol. $m^{-2}.s^{-1}$. The plants were allowed to equilibrate in the phytotron for one week prior to the start of the treatment. The RWC (B.16.1) as well as the water potential (WP) (B.16.2) were calculated. At intervals the leaf samples were removed, wrapped in tinfoil, flash frozen in liquid nitrogen and then stored at –70°C. Usually one leaf was used per sampling interval. The samples for calculating RWC and WP were removed simultaneously and sealed immediately in an air-tight container. For all the treatments a sample was removed from the plant immediately before the stress was imposed and unless otherwise specified, at intervals of 6, 12 and 24 hours for the first 24 hours and then once every 24 hours for seven days. For experiments in which leaf explants were used a water control was included to ensure that any response observed was not a spurious result due to wounding.

Heat treatment

All conditions within the phytotron were as described above except that the plant was subjected to an elevated temperature of 42°C for the duration of the treatment. The plant was watered once a day to prevent dehydration.

Dehydration treatment

The plant was allowed to dry naturally by witholding water. The drying was monitored visually and samples were removed from the plant at arbitrary intervals until fully dehydrated. At this point the plant was watered and allowed to recover and samples were removed daily until the plant was fully hydrated.

High light treatment

All conditions in the phytotron were as described above except for the light intensity which was 1500 μMol. $m^{-2}.s^{-1}$. Again the plant was watered throughout the duration of the treatment.

Cold treatment

An *X. viscosa* plant was placed in a cold room at 4°C. The light intensity was 280 μMol.m^{-1}.s^{-1} with a light dark cycle of 16hours/8hours. The plant was well watered throughout the duration of the cold stress.

Salt treatment

An *X. viscosa* plant was allowed to equilibrate in the phytotron for one week after which leaf samples were removed and submerged in a 150 mM NaCl solution (B.14.10) in a 50 ml Sterilin tube (Bibby Sterilin, England). Three leaves were placed in the Sterilin tube containing 20 ml of the salt solution. The base of the leaves were cut with a sterile blade while submerged in the salt solution to prevent air bubbles from entering the leaves' veins and blocking the uptake of the solution. The lid of the tube was placed at an angle on top of the tube so that the leaves could protrude and receive light while minimising evaporation. At the specified time points, the leaves were removed from the solution, excess solution blotted off and frozen. A water control was conducted simultaneously in the same manner, except that the tubes contained sterile distilled water instead of the salt solution.

ABA treatment

Leaf samples were submerged in 20 ml of a 100 μM ABA solution (Sigma-Aldrich, UK; B.14.1) in a 50 ml Sterilin tube. The tubes were covered in tinfoil to minimise light inactivation of ABA. This treatment was conducted simultaneously to and in the same manner as the salt treatment and the same water control was used.

3.2.3. Northern blot analysis

All solutions used for RNA extractions were treated with 0.01% diethylpyruvocarbonate (DEPC; B14.4) and all Eppendorf tubes and pipette tips were autoclaved twice. Total RNA was isolated from samples using Trizol Reagent (Life Technologies, USA) according to the manufacturer's specifications. Approximately 100 mg leaf tissue was used per extraction. The RNA concentrations were quantified spectrophotometrically at a wavelength of 260 nm (A.1.3.1). RNA was electrophoresed on 1.2% TBE gels (A.1.2.2) and transferred to Hybond XL nylon membranes (Amersham, UK) by capillary elution (A.1.4.3).

A 1-kb region of the C-terminal domain of the *Grp94* gene was PCR amplified from pGEMT-Grp94 (C.2.1) to be used as a probe. The reaction (A.1.6.4.1) was performed with the forward primer, RTFor (C.1.6), and the reverse primer, Hsp90Rev3`A (C.1.2). The PCR reaction was performed using the following protocol: (1) 94°C for 3 minutes (2) 94°C for 30

seconds (3) 55°C for 30 seconds (4) 72°C for 1 minute (5) 72°C for 7 minutes. Steps 2-4 were repeated 25 times. The resultant product was electrophoresed on an 0.8% TBE gel and the 1-kB band was cut out of the gel, purified (A.1.6.1) and labelled with [^{32}P]dCTP by PCR-labeling (A.1.6.4.2) with the same primers as above. The labelled PCR product was purified through a Sephadex G-50 column (A.1.6.3) and used as a probe for the Northern hybridization reaction (A.1.4.4)

3.2.4. Western blot analysis

Total protein was extracted from each of the samples from the treatments according to the method of Bartels *et al.* (1991) with modifications (A.2.1). For each extraction 100 mg of leaf material was used, however, 20 mg of leaf material was used for the dehydrated samples. Proteins were separated on pairs of 10% denaturing polyacrylamide gels (A.2.2). One of each pair was stained with Coomassie blue (A.2.4), while the other was used for western transfer onto Osmonics nitrocellulose membrane (0.45 μm pore size) (A.2.3). The membrane was probed with madagascar periwinkle Grp94 antiserum (1:1000), kindly donated by G. Schroeder. The antibody was raised against a protein encoded by the internal 1.5-kb fragment of the *Grp94* from *Catharanthus roseus* (Schroeder *et al.*, 1993). This antibody was thought to be suitable as sequence alignments showed that the *X. viscosa* Grp94 protein was most similar to that of *C. roseus* (Figure 2.6, Chapter 2). Detection was performed using goat anti-rabbit antibody conjugated to horse-radish peroxidase (Sigma-Aldrich, UK) (A.2.5).

3.3. RESULTS AND DISCUSSION

3.3.1. Northern blot analysis

RNA was originally extracted from a healthy, hydrated *X. viscosa* plant (approximately 90% RWC) and probed with the 3`-terminal region of the Grp94 cDNA to confirm the size of the transcript (Figure 3.1). Analysis of the northern blot revealed that the transcript is 2.8 kb (Figure 3.2).

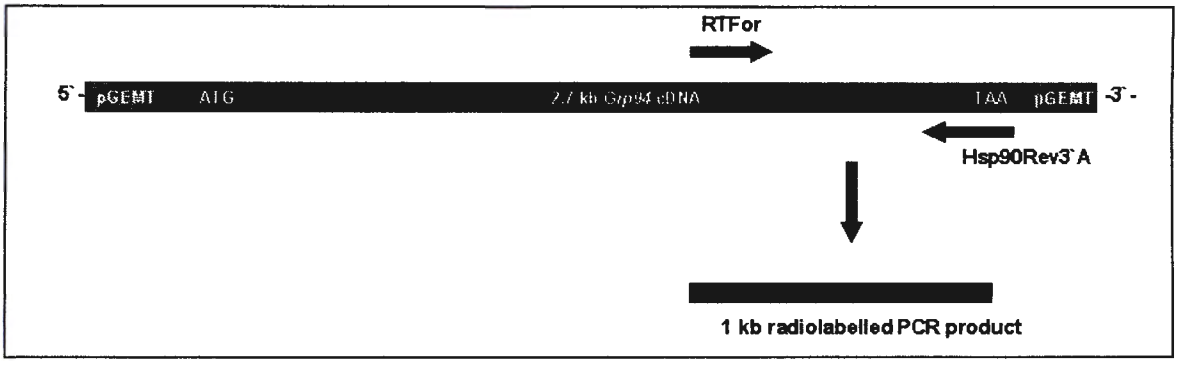


Figure 3.1: The method employed for the preparation of the radiolabelled probe for northern blot analysis. A 1-kb region at the 3`end of the cDNA was PCR labelled with [32P]dCTP. The primers used (RTFor and Hsp90Rev3`A) are included in the diagram.

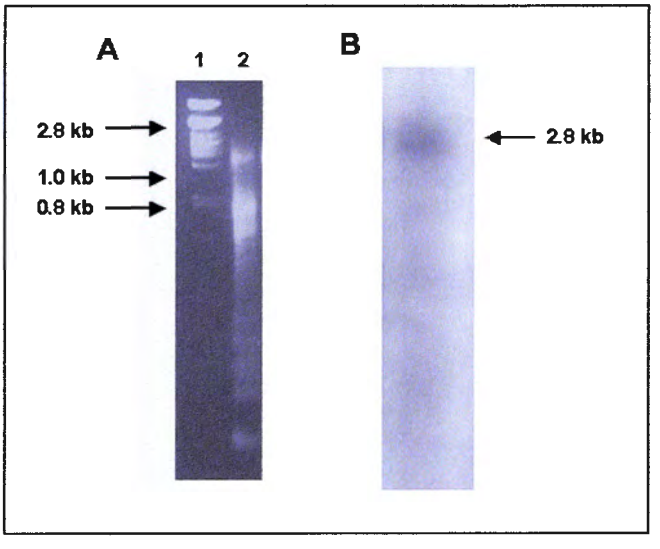


Figure 3.2: Northern blot analysis to determine the size of the *XVGrp94* transcript. A. RNA gel; Lanes, 1 λ *Pst* I DNA markers; 2, 90% RWC RNA. 10 μg of RNA was electrophoresed on a 1.2% TBE gel and transferred onto a nylon membrane. Arrows on the left hand size indicate molecular sizes determined from the λ *Pst* I markers. B. Northern blot. A 1-kb region of the 3`terminus of the cDNA was used as a probe.

The same protocol was then used to determine the expression of the gene in response to various treatments conducted on the plants. The RNA extracted from heat-treated *X. viscosa* leaves was probed with the 3`-terminal region of the cDNA. The RNA was of good quality and quantity (Figure 3.3A), however, the 2.6-kb band could not be detected even though the same low stringency washes were used as for the initial blot to determine transcript size (Figure 3.3B). The RNA extraction, electrophoresis, transfer and hybridisation were repeated and the same results were observed. This was unexpected as XVGrp94 is a putative heat shock protein and an increase in its mRNA levels in response to elevated temperatures was anticipated. The RNA from a previous dehydration treatment (performed by Shaheen Mowla) was also probed with the cDNA and the same results were obtained

(not shown). Again this was unexpected as the cDNA was originally isolated from mRNA extracted from a dehydrated plant (Mundree, 2000). Other more sensitive methods of mRNA detection, such as quantitative RT-PCR or RNAse protection assays, may reveal more about the mRNA levels during the imposed stresses. As it was not possible to determine why the northern analysis results were negative, it was decided to investigate the expression of the protein itself in response to the various stresses.

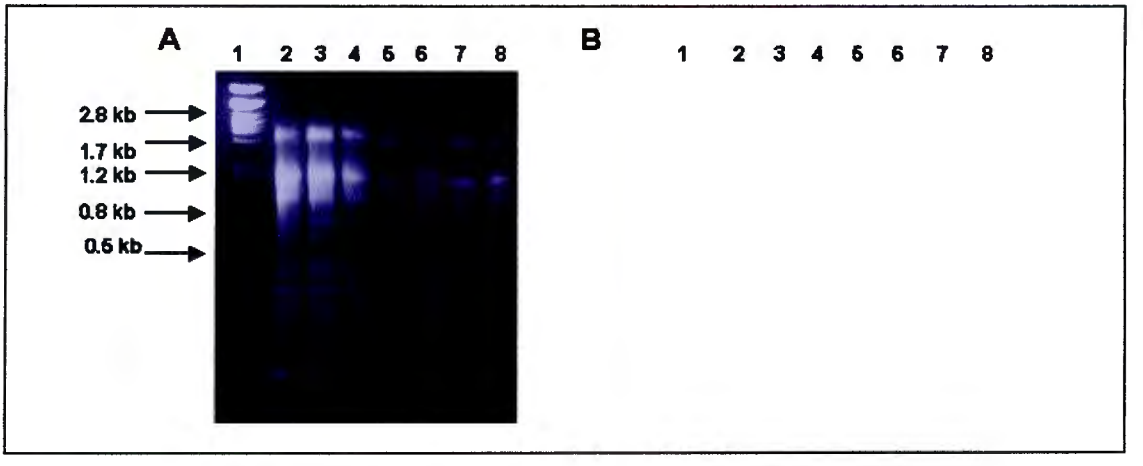


Figure 3.3: Northern blot analysis of RNA isolated from heat-stressed *X. viscosa*. A. RNA gel. Lanes 1, λ *Pst* I DNA markers; 2, 0 hours; 6 hours; 4, 12 hours; 5, 24 hours; 6, 48 hours; 7, 72 hours; 8, 96 hours. Arrows on the left hand side indicate molecular sizes determined from the λ *Pst* I markers. B. Northern blot. The RNA was probed with the 3`end of the cDNA.

3.3.2. Western blot analysis

X. viscosa was subjected to a number of environmental stresses in order to determine the expression pattern of the Grp94 protein by western analysis (Figures 3.4. to 3.12). The Coomassie-stained gels showed that the loading of the samples was approximately equal. Protein concentrations could not be determined using the Bradford quantification assay as the extraction buffer contained β-mercaptoethanol which interferes with the assay. Equal loading of protein was therefore determined by visualisation of Coomassie stained gels. After the completion of transfer, staining of the gels and membranes with Coomassie blue and Ponceau S respectively showed that the transfer in all cases was successful and all the proteins transferred efficiently.

The relative water content and water potential of the plant was determined at each sample time point for the various treatments and the results of these are presented together with the corresponding western blots (Figures 3.6. to 3.12). The antibody used in this study was raised against the Grp94 from *C. roseus* (Schroeder *et al.*, 1993), therefore as a positive control *C. roseus* proteins were extracted and probed with the antibody. A separate gel was

control *C. roseus* proteins were extracted and probed with the antibody. A separate gel was run as earlier attempts showed the reaction between the positive control and the antibody was so strong that no reaction could be observed with the *X. viscosa* Grp94 (Figure 3.4).

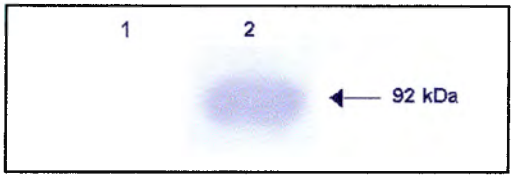


Figure 3.4: Western blot analysis of total protein extracts from *X. viscosa* leaves (lane 1) and *C. roseus* leaves (lane 2). 10 µl of each sample was run a 10% polyacrylamide gel, transferred to nitrocellulose membrane and probed with anti-Grp94 raised against Grp94 from *C. roseus* (Schroeder *et al.*, 1993). The arrow on the right hand side represents molecular weight as determined from comparison with Coomassie blue-stained gel containing molecular weight markers.

A western blot was conducted on proteins extracted from heat-treated leaves using the rabbit pre-immune serum as a negative control (Figure 3.5). This showed that there was no spurious cross-reactivity between the naïve rabbit serum and the XVGrp94 protein. There was slight evidence of cross-reactivity with a 55-kDa protein, but it was not a strong reaction and it was not detected in the western blots probed with the Grp94 antibody (see below).

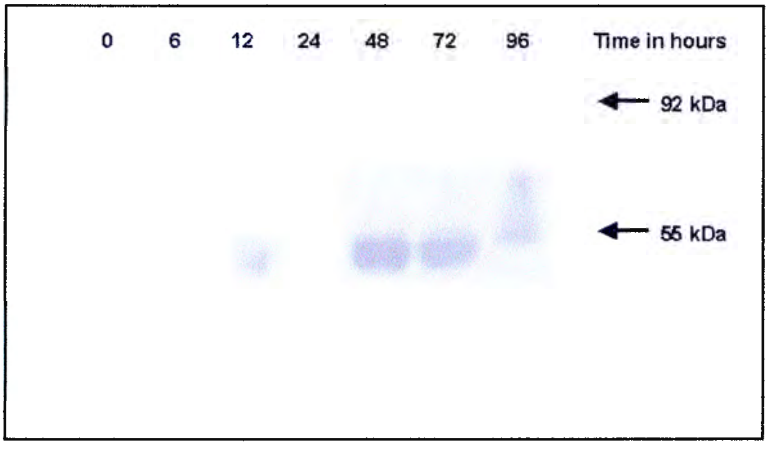


Figure 3.5: Western blot analysis of total protein extracts from leaves of a heat-treated *X. viscosa* Lanes 1, 0 hours; 2, 6 hours; 3, 12 hours; 4, 24 hours; 5, 48 hours; 6, 72 hours; 7, 96 hours. 10 µl of each sample was electrophoresed on a 10% polyacrylamide gel, transferred to nitrocellulose membrane and probed with the rabbit pre-immune serum (Schroeder *et al.*, 1993). Arrows on the right hand side represent molecular weights as determined from comparison with Coomassie blue-stained gel containing molecular weight markers.

A significant increase in XVGrp94 protein was observed during heat stress, as expected for a heat shock protein (Figure 3.6). The increase was fairly rapid with the protein levels rising within 6 hours of commencement of the stress. The level continued to rise throughout the treatment and at the end of the experiment there was no evidence of a decline in the level of XVGrp94 (96 hours).

During conditions of stress, such as high temperature, proteins are denatured and there is an increase in exposed hydrophobic domains (Hendrick and Hartl, 1993; Morimoto *et al.,* 1994, Hartl., 1996; Miernyk, 1999). Molecular chaperones recognise and bind to these residues, protecting the protein as it unfolds and thereby preventing aggregation (Ellis and van der Vies, 1991, Gething and Sambrook., 1992; Parsell and Lindquist, 1993; Morimoto *et al.,* 1994; Hartl, 1996; Netzer and Hartl., 1998; Miernyk, 1999). As the heat stress continues a greater proportion of the cellular protein becomes denatured and damaged thus increasing the demand for protection. As the levels XVGrp94 were seen to increase during the heat stress this suggests a protective role for this protein. Interestingly it has begun to emerge that Hsp90 itself does not refold proteins, but rather it binds to unfolded proteins and holds them in a state ready to be folded with the aid of other chaperones (Jakob *et al.,* 1995; Freeman and Morimoto, 1996; Yonehara *et al.,* 1996; Nathan *et al.*, 1997). Thus it could be suggested that more of the XVGrp94 protein is required as proteins become denatured, to bind and protect the damaged proteins until they can be refolded. Also, it is thought that this protein may be located within the ER (see Chapter 2), and it may be involved in protecting misfolded proteins accumulating within the ER during stressful conditions. In this way XVGrp94 could prevent protein aggregation and once normal metabolism resumes the proteins can be refolded without any further consequence as a result of aggregation.

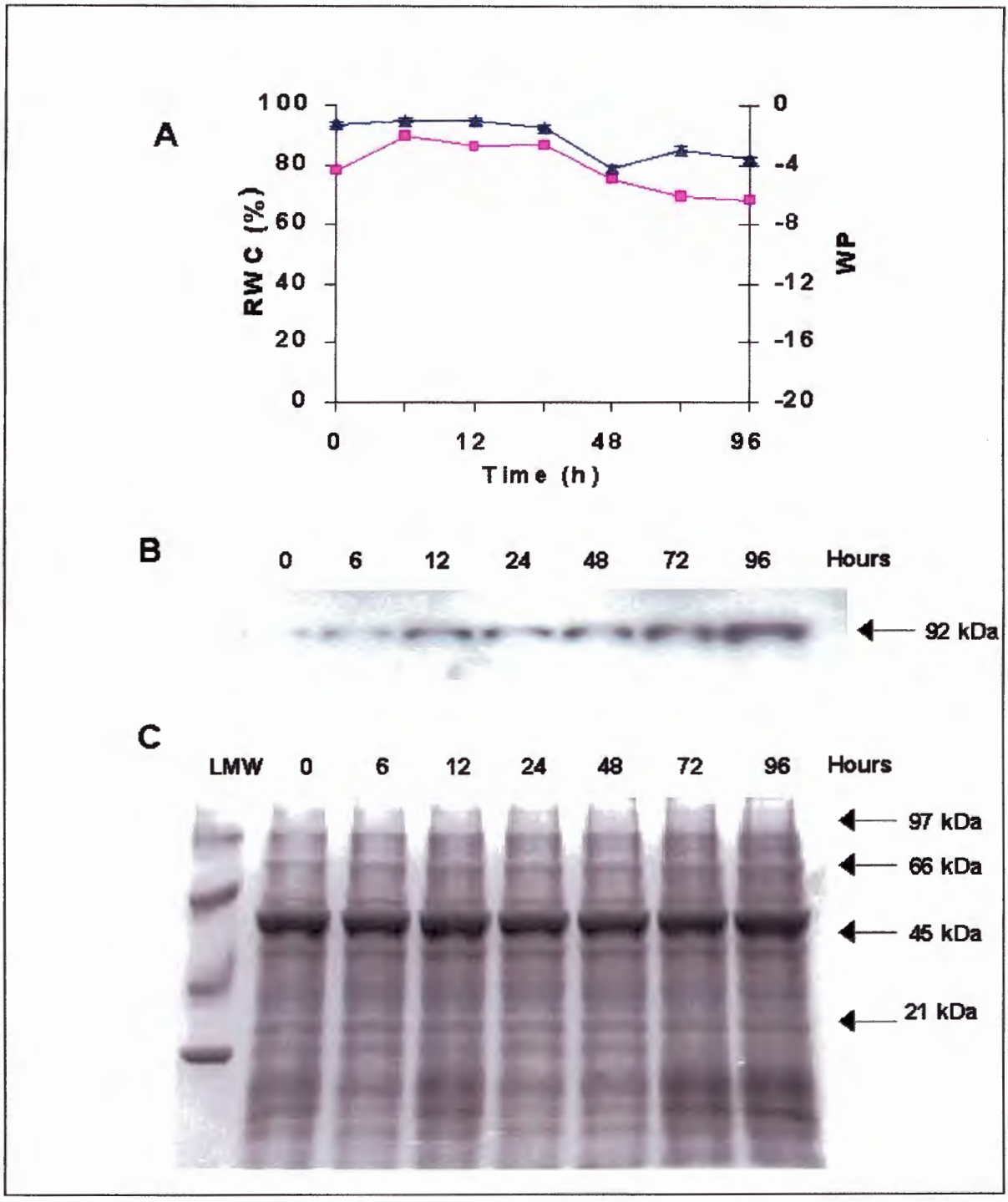


Figure 3.6: Analysis of total protein extracts from leaves of a heat-treated *X. viscosa*. A. Graph of the relative water content (RWC) (▲) and water potential (WP) (□) versus time. Data for RWC are means ± Standard deviation, $n = 2$. B. Western blot of total *X. viscosa* proteins. The membrane was probed with anti-Grp94 raised against the Grp94 from *C. roesus* (Schroeder *et al.*, 1993). C. 10% polyacrylamide gel of protein samples extracted from *X. Viscosa* leaves. LMW, low molecular weight marker; Time (in hours) is indicated on the gel photograph. 10 µl of each sample was loaded, electrophoresed and transferred onto nitrocelluolose membrane. Arrows on the right hand side indicate molecular sizes determined from the molecular weight markers.

During dehydration stress (Figure 3.7B) an increase in XVGrp94 was also observed. Although there was slight evidence of the protein at 94% RWC, this is not unusual as Hsp90 had been found to be an abundant protein constituting 1% of the total cellular protein under normal cellular conditions (Gething and Sambrook, 1992; Parsell and Lindquist, 1993; 1994). However as the stress progressed and the plant became increasingly dehydrated there was a marked increase in the amount of XVGrp94 protein present, with significant amounts of the protein being produced between 50% to 35% RWC with further increase as the plant dried down to 7% RWC. As the plant rehydrated (Figure 3.7C), the amount of XVGrp94 protein decreased until it reached similar levels to that observed before the plant had begun dehydrating (Figure 3.7B). This suggests a role for this protein during the dehydration stress. During conditions of dehydration, as water leaves the cell, proteins become damaged and unfold and may interact with other proteins or cellular components. The cellular components also become more concentrated increasing the chances of aggregation of proteins. XVGrp94 may act to prevent aggregation of unfolded proteins by binding to and stabilising them thereby reducing any further damage (Gething and Sambrook, 1992; Hendrick and Hartl, 1993; Morimoto *et al.,* 1994; Parsell and Lindquist, 1994; Boston *et al.,* 1996; Hartl, 1996; Johnson and Craig, 1997; Netzer and Hartl, 1998). Desiccation stress also results in the chemical modification of protein residues (e.g. deamination or oxidation) and these proteins must be degraded or repaired to ensure the survival of the plant (Ingram and Bartels, 1996). While there are enzymes to degrade or repair these proteins (Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1997), XVGrp94 may also have a role in this process by binding to and stabilising these modified proteins until the proteins required for repair or degradation are available. In this manner XVGrp94 may reduce any further damage that may occur if these damaged proteins are left unchecked.

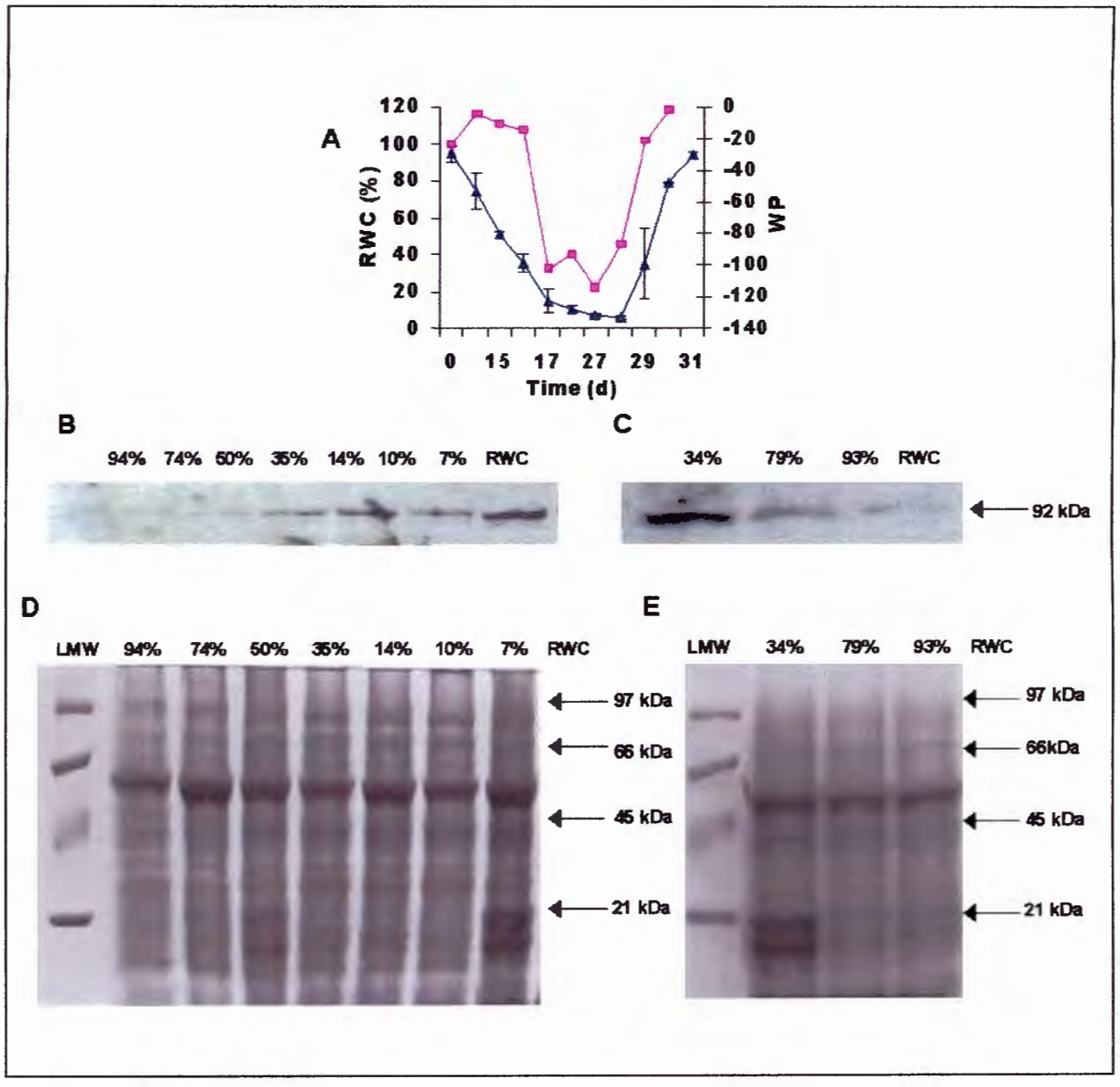


Figure 3.7: Analysis of total protein extracts from dehydrated *X. viscosa* leaves. A. Graph of the relative water content (RWC) (▲) and water potential (WP) (■) versus time. The graph includes the dehydration as well as the rehydration data. Data for RWC are means ± Standard deviation, $n = 2$. B. Western blot of total proteins extracted from a dehydrating *X. viscosa*. C. Western blot of total *X. viscosa* proteins extracted from leaves of a rehydrating plant. The membranes were probed with anti-Grp94 raised against the Grp94 from *C. roesus* (Schroeder *et al.*, 1993). D. 10% polyacrylamide gel of total protein from leaves from a dehydrating *X. viscosa* plant. E. 10% polyacrylamide gel of total protein from leaves of a rehydrating *X. viscosa* plant. For both gels 10 µl of each sample was loaded, electrophoresed and transferred onto nitrocelluolose membrane. Arrows on the right hand side indicate molecular sizes determined from the molecular weight markers.

There was no observable increase in Grp94 in response to the cold stress (Figure 3.8). These results would suggest that XVGrp94 does not have a role in cold temperature acclimation, but rather, as observed with the heat stress, it probably protects against damage that may occur as a result of the stress.

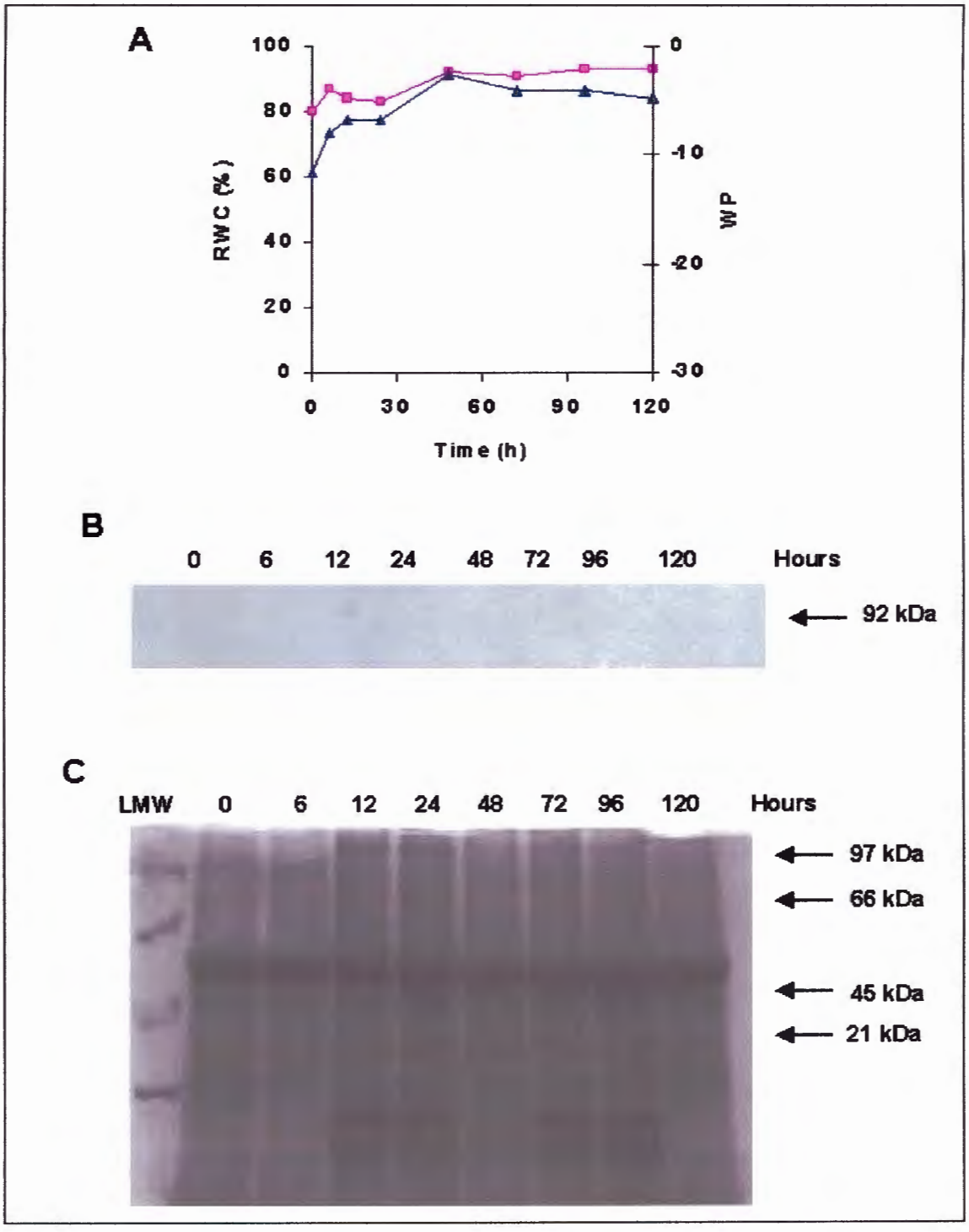


Figure 3.8: Analysis of total protein extracts from cold-treated *X. viscosa* leaves. A. Graph of the relative water content (RWC) (▲) and water potential (WP) (■) versus time. Data for RWC are means ± Standard deviation, $n = 2$. B. Western blot of total *X. viscosa* proteins extracted from leaves. Time (in hours) is indicated on the photograph. The membrane was probed with anti-Grp94 raised against the Grp94 from *C. roesus* (Schroeder *et al.*, 1993). C. 10% polyacrylamide gel of total protein from leaves of a *X. viscosa* plant. LMW, molecular weight markers; Time (in hours) is indicated on the gel photograph. 10 µl of each sample was loaded, electrophoresed and transferred onto nitrocelluolose membrane. Arrows on the right hand side indicate molecular sizes determined from the molecular weight markers.

There was also no response to high light (Figure 3.9) or salt (Figure 3.10), possibly because the stresses imposed did not result in sufficient protein damage.

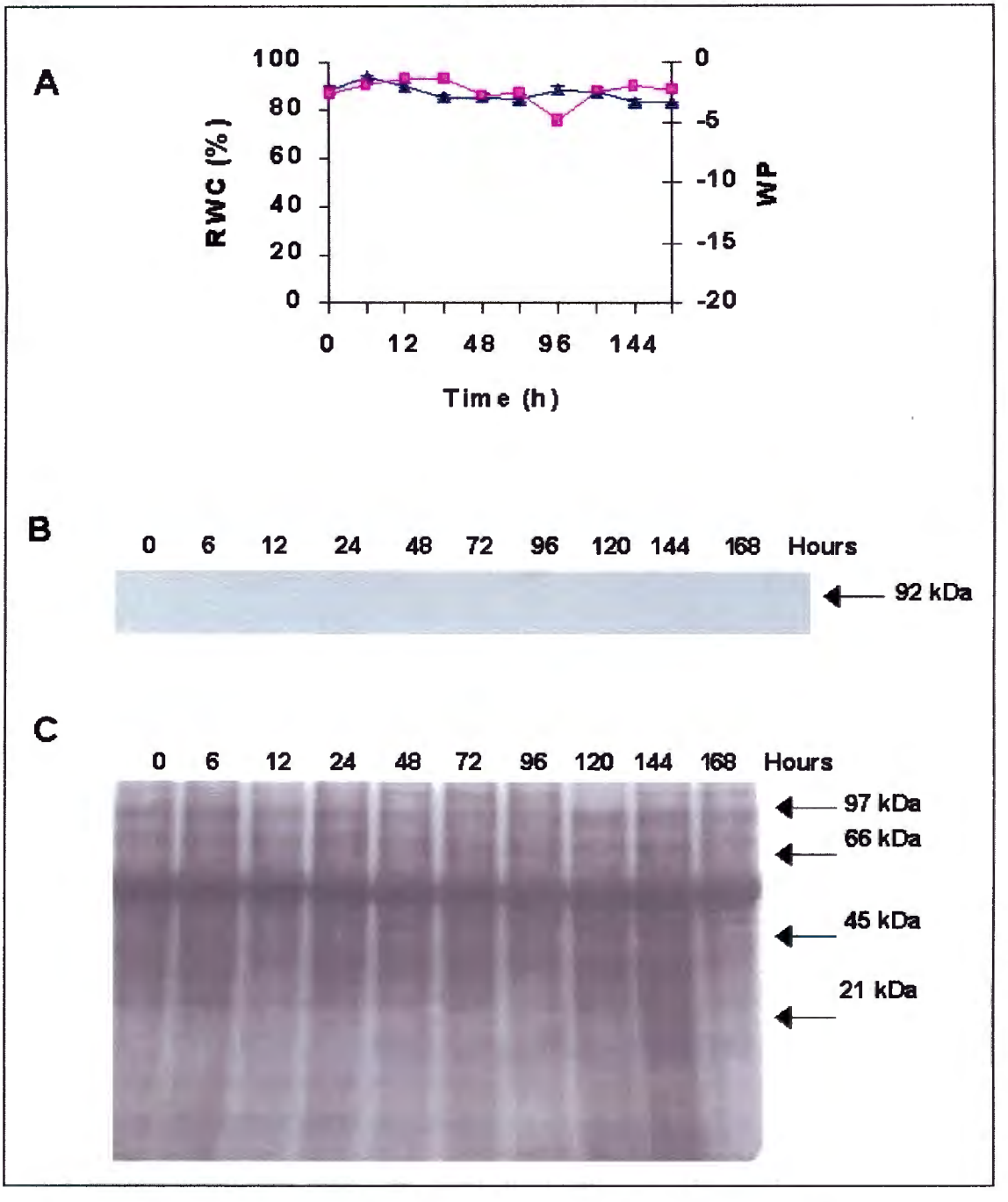


Figure 3.9: Analysis of total protein extracted from leaves of an *X. viscosa* plant subjected to high light (1500 μMol. M-2.s-1). A. Graph of the relative water content (RWC) (▲) and water potential (WP) (■) versus time. Data for RWC are means ± Standard deviation, *n* = 2. B. Western blot of total *X. viscosa* proteins extracted from leaves. Time (in hours) is indicated on the photograph. The membrane was probed with anti-Grp94 raised against the Grp94 from *C. roesus* (Schroeder *et al.*, 1993). C. 10% polyacrylamide gel of total protein from *X. viscosa* leaves. LMW, molecular weight markers; Time (in hours) is indicated on the gel photograph. 10 μl of each sample was loaded, electrophoresed and transferred onto nitrocelluolose membrane. Arrows on the right hand side indicate molecular sizes determined from the molecular weight markers.

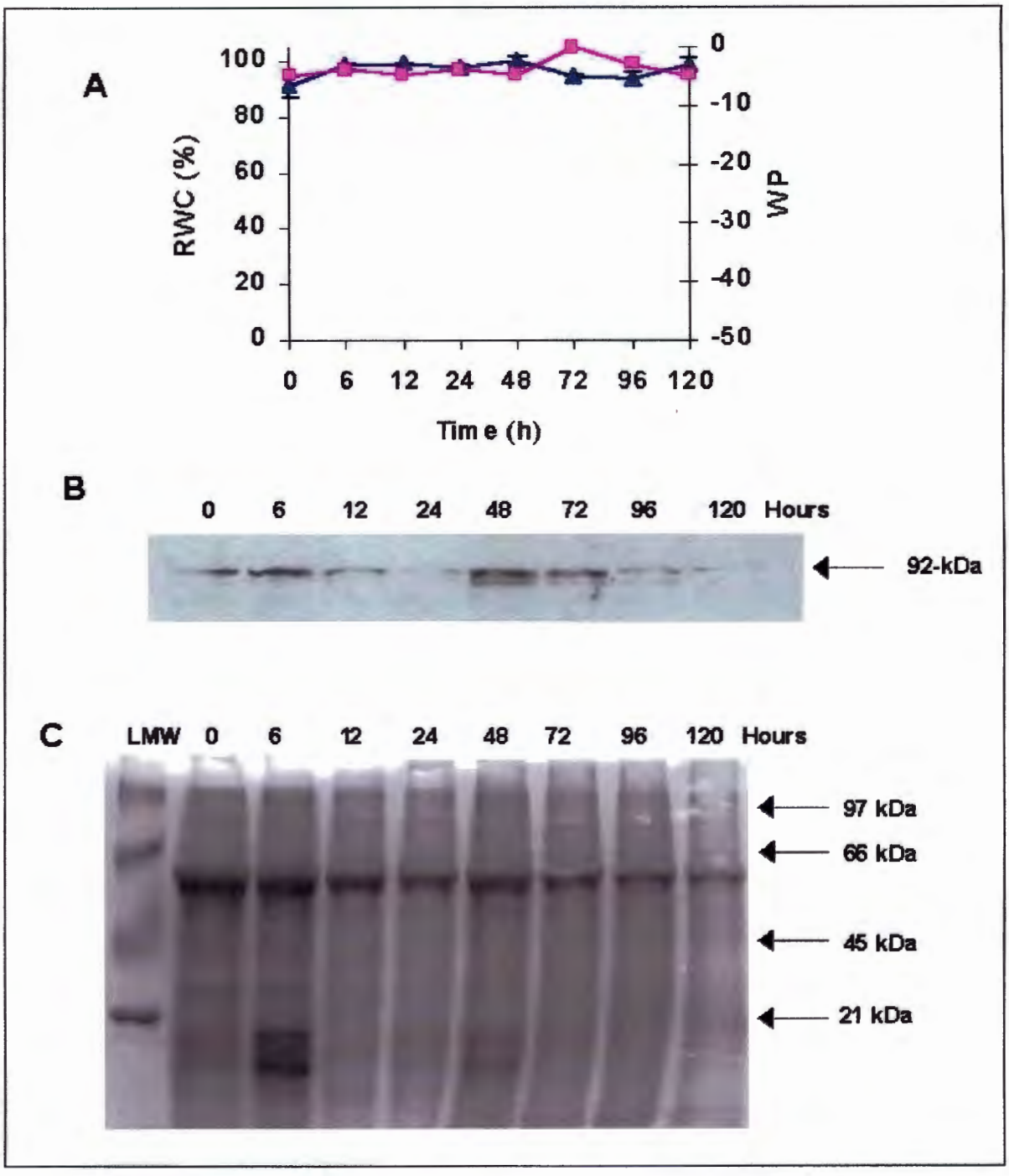


Figure 3.10: Analysis of total protein extracts from leaves of a salt-treated *X. viscosa* plant. A. Graph of the relative water content (RWC) (▲) and water potential (WP) (□) versus time. Data for RWC are means ± Standard deviation, $n = 2$. B. Western blot of total *X. viscosa* proteins. Time (in hours) is indicated on the photograph. The membrane was probed with anti-Grp94 raised against the Grp94 from *C. roesus* (Schroeder *et al.*, 1993). C. 10% polyacrylamide gel of total protein from leaves of a *X. viscosa* plant. LMW, molecular weight markers; Time (in hours) is indicated on the gel photograph. 10 µl of each sample was loaded, electrophoresed and transferred onto nitrocelluolose membrane. Arrows on the right hand side indicate molecular sizes determined from the molecular weight markers.

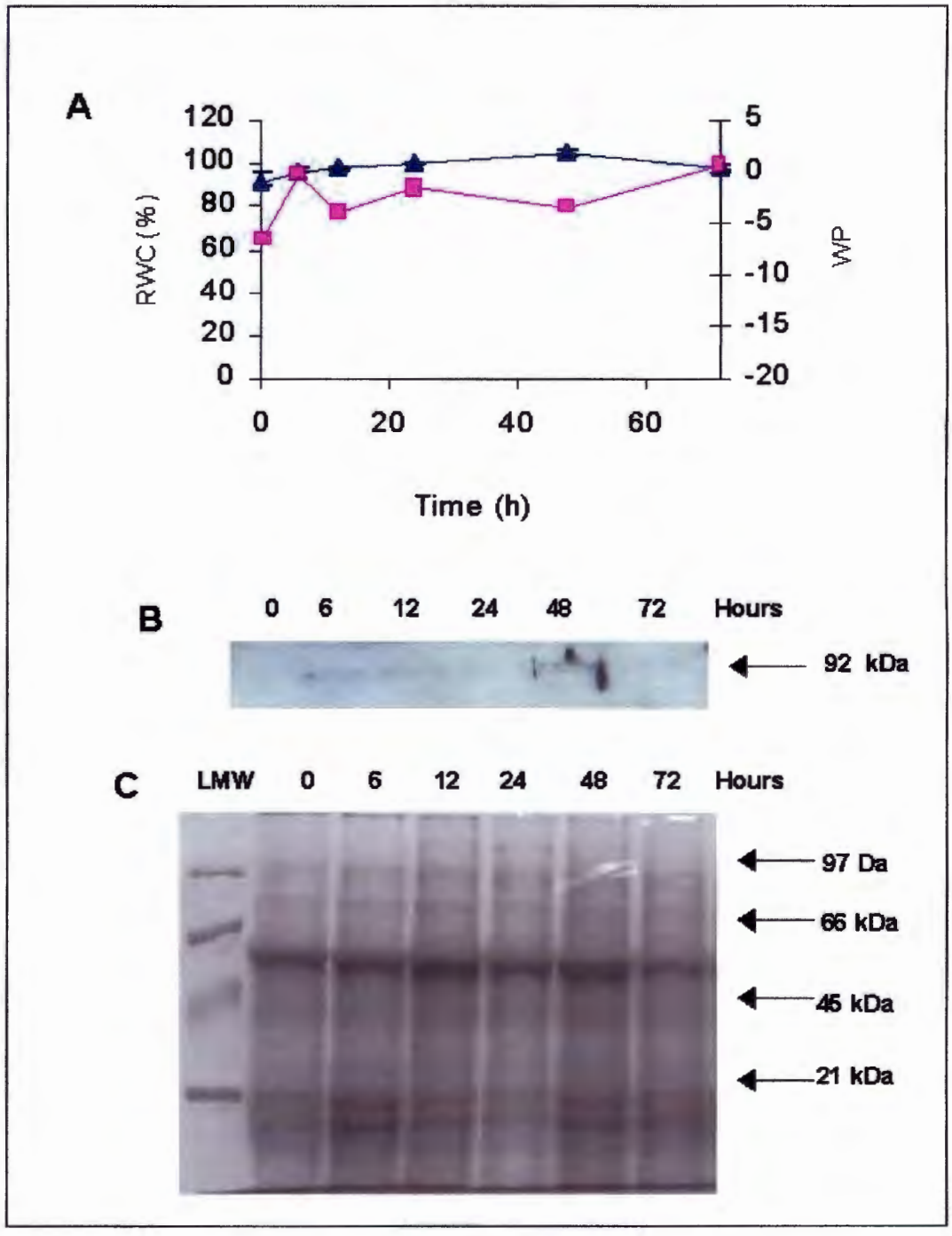


Figure 3.11: Analysis of total protein extracts from ABA-treated leaves of *X. viscosa*. A. Graph of the relative water content (RWC) (▲) and water potential (WP) (■) versus time. Data for RWC are means ± Standard deviation, $n = 2$. B. Western blot of total *X. viscosa* proteins. Time (in hours) is indicated on the photograph. The membrane was probed with anti-Grp94 raised against the Grp94 from *C. roesus* (Schroeder *et al.*, 1993). C. 10% polyacrylamide gel of total protein from leaves of a *X. viscosa* plant. LMW, molecular weight markers; Time (in hours) is indicated on the gel photograph. 10 µl of each sample was loaded, electrophoresed and transferred onto nitrocelluolose membrane. Arrows on the right hand side indicate molecular sizes determined from the molecular weight markers.

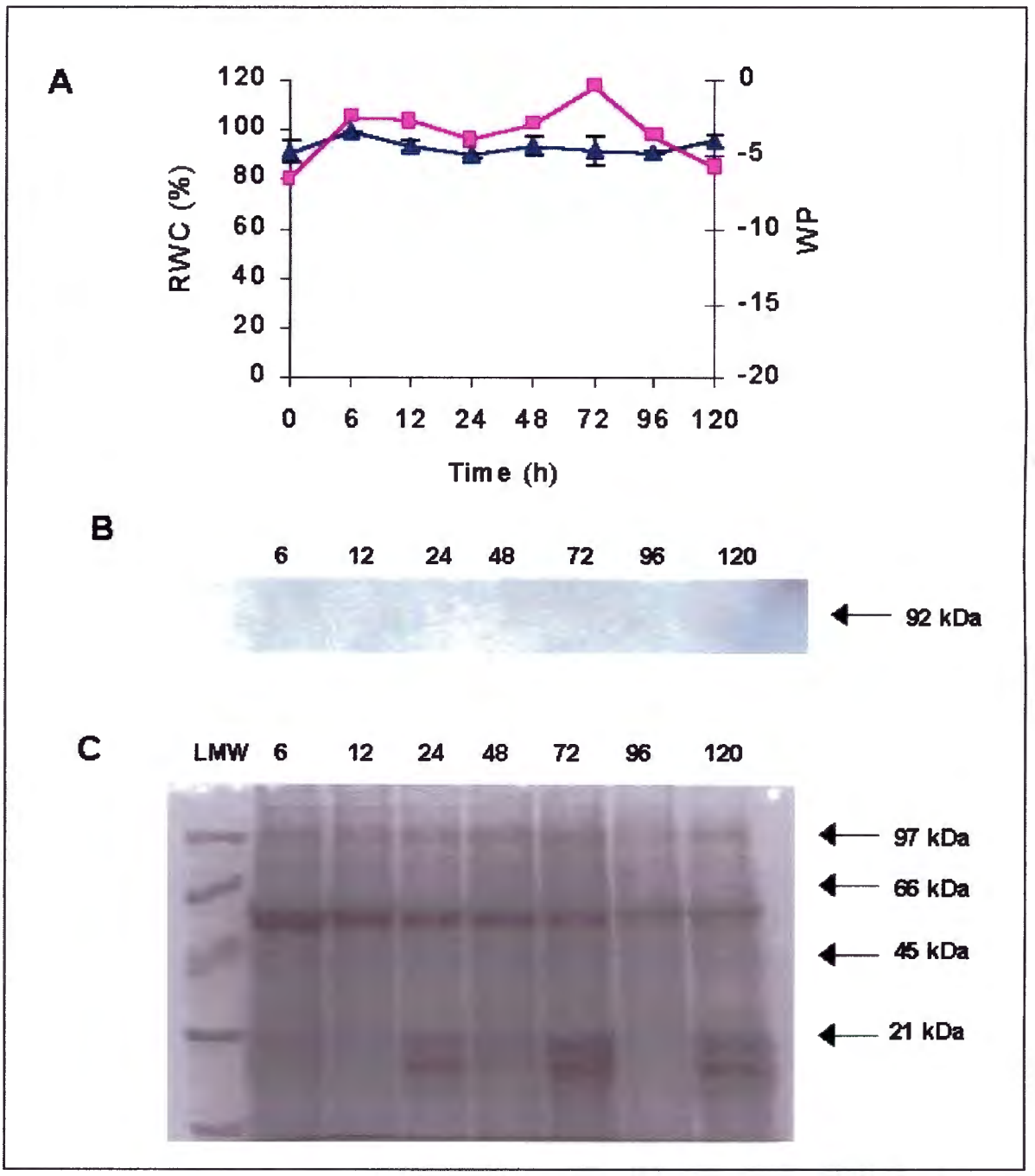


Figure 3.12: Analysis of total protein extracted from *X. viscosa* leaves included as a water control. A. Graph of the relative water content (RWC) (▲) and water potential (WP) (■) versus time. Data for RWC are means ± Standard deviation, $n = 2$. B. Western blot of total *X. viscosa* proteins. Time (in hours) is indicated on the photograph. The membrane was probed with anti-Grp94 raised against the Grp94 from *C. roesus* (Schroeder *et al.*, 1993). C. 10% polyacrylamide gel of total protein from leaves of a *X. viscosa* plant. LMW, molecular weight markers; Time (in hours) is indicated on the gel photograph. 10 µl of each sample was loaded, electrophoresed and transferred onto nitrocelluolose membrane. Arrows on the right hand side indicate molecular sizes determined from the molecular weight markers.

There was no response to ABA (Figure 3.11), suggesting that the *XVGrp94* gene may not be regulated by ABA. However, it remains to be determined whether the plant had actually taken up the ABA solution. This could be achieved by probing with an antibody raised against a protein known to be induced by ABA, or to probe with a gene that is known to respond to ABA. Alternatively, the ABA content of the leaf prior to the exogenous ABA application could be measured. For both the salt and ABA treatments, water controls were conducted simultaneously and no increase in the protein was observed due to wounding (Figure 3.12).

3.4. CONCLUSION

The failure of the Northern blot analysis is difficult to explain. Possible reasons could include: inadequate transfer due to the large size of the transcript, suboptimal hybridization and washing conditions or inadequate labeling of the probe. These possibilities could be tested by the inclusion of a positive control, which was not performed in this case. The failure of the Northern blots prompted the analysis of the protein levels under various stresses by western blot analysis.

The results of the western blot analysis indicate an increase in XVGrp94 during conditions of stress such as high temperature and dehydration. XVGrp94 may have a role, possibly as a chaperone, as its expression is upregulated during conditions that result in protein denaturation and damage. This idea could be supported by functional studies with the recombinant XVGrp94, which would be expected to show protection of a substrate protein.

If XVGrp94 is located within the ER, a requirement for increased levels of the protein during conditions of stress is obvious as malfolded and damaged proteins requiring repair or degradation will accumulate within the ER. Similarly proteins within the cytosol are also damaged during the stress and will need to be protected from aggregation until they can be repaired. Tunicamycin, an inhibitor of co-translational N-glycosylation results in increased expression of ER-located heat shock proteins (Kozutsumi *et al.*, 1988; D'Amico *et al.*, 1992), and as XVGrp94 is thought to be located within the ER, it would be of interest to test the effects of that inhibitor on the expression of the protein.

CHAPTER FOUR

EXPRESSION OF *XVGrp94* IN *E. coli*

SUMMARY

The full-length *XVGrp94* cDNA was found to contain a TAG stop codon 18 nucleotides upstream of the putative start codon. This stop codon would prevent the expression of XVGrp94 in the pPro EX HT^{TM} expression system. The stop codon is located downstream of the histidine tag and just upstream of the beginning of the XVGrp94 sequence which would result in the termination of translation just after the histidine tag. The 2.45-kb *XVGrp94* open reading frame (ORF), from the putative ATG to the TAA translation stop, was PCR amplified from pGEMT-XVGrp94 and cloned into the pProEX HTa expression vector. The resultant construct was used to transform *E. coli* and expression of the recombinant protein was induced by the addition of IPTG. A small-scale induction was performed initially, and expression of the 94-kDa recombinant protein was observed within the first hour of induction and the levels remained high throughout the three-hour induction period. A large-scale induction was performed and expression of the recombinant protein was allowed to proceed for three hours. Purification of the recombinant protein on Ni-NTA resin was unsuccessful and it was found that the recombinant protein was insoluble.

4.1. INTRODUCTION

The pProEX HT expression system allows high-level expression of foreign proteins in *E. coli*. Additionally, the system incorporates a 6X-histidine residue tag at the N-terminus of the protein which allows for its purification on histidine affinity columns. Three different vectors, pProEX HTa, b and c are available, each with the 6X histidine tag in a different reading frame relative to the multiple cloning site to ensure that an in-frame fusion is always possible. The gene of interest is cloned into the appropriate vector, and the addition of IPTG results in expression of the protein from the *Trc* promoter. After purification, the 6X histidine tag can be removed by a TEV protease, whose recognition site is encoded in the cloning vector between the sequence encoding the 6X histidine tag and the multiple cloning site (Life Technologies, USA).

The 6X histidine tag is 0.84 kDa and is much smaller than most other affinity tags, including the 26-kDa glutathionine-S-transferase, the 30-kDa protein A and the 40-kDa maltose binding protein (QIAexpress Manual, Qiagen). The 6X His tag is also uncharged at physiological pH and it rarely interferes with protein structure or function and does not contribute to protein immunogenicity nor does it interfere with protein secretion (Sisk *et al.*, 1994).

Recombinant proteins containing the histidine tag can be purified using a technique called immobilized metal affinity chromatography (IMAC) (Petty, 1996; Schmidbauer & Strobel, 1997). A metal chelating group is immobilised on a chromatographic medium, and a multivalent metal ion, such as Cu^{2+}, Ni^{2+}, Zn^{2+} or Co^{2+}, is bound such that some coordination sites remain available for selective interaction with proteins. The histidine tag, fused to the recombinant protein, specifically interacts with the chelated ions, thereby holding the protein on the medium. Other components bind weakly, if at all. The fusion protein is eluted by reducing the pH which causes the histidine residues to become protonated and to dissociate from the medium. Alternatively elution can be achieved by increasing the levels of a competitive eluting agent, such as imidazole (Janknecht et al., 1991; Petty, 1996; Schmidbauer and Strobel, 1997; QIAexpress manual, Qiagen).

The QIAexpress Ni-NTA (nickel-nitrilotriacetic acid) purification system is a convenient and simple method for the isolation of recombinant 6X His-tagged proteins and peptides. This system exploits the four binding sites of NTA to bind nickel ions more tightly than other metal-chelating purification systems which only have three sites available for interaction with metal ions. This results in a greater binding capacity and minimizes non-specific binding thus leading to purer protein preparations (QIAexpress manual, Qiagen). This system allows the purification of almost any 6X His-tagged proteins suitable for any applications including: (1) purification of functional, conformationally active proteins (Siska et al., 1994); (2) purification under denaturing conditions for antibody production (Petty, 1996); (3) crystallization for determination of three-dimensional structure and; (4) assays involving protein–protein and protein–DNA interactions (QIAexpress manual, Qiagen).

This chapter describes an attempt to express the recombinant XVGrp94 protein in *E. coli* and purify it using Ni-NTA resin. This was performed with the aim of conducting functional studies with the recombinant XVGrp94 protein. However this was not possible as despite successfully expressing the recombinant protein in *E. coli*, purification was unsuccessful.

4.2. MATERIALS AND METHODS

4.2.1. Strains and plasmids

All plasmid constructions and protein expression experiments were conducted using either *E. coli* DH5α or *E. coli* JM109. The *E. coli* cultures were grown and maintained on LA (B.15.1.1) at 37°C, with the appropriate antibiotic selection. For growth in liquid culture, LB appropriate antibiotics were used. All plasmids used in these experiments are listed in Table 4.1. The pProEX™ HT series of vectors (Life Technologies, USA) differ from each other with respect to their reading frames relative to the 6x histidine affinity tag.

Table 4.1 Plasmids used in this work

Plasmid	Relevant Characteristics	Source
pProEX Hta	6x histidine affinity tag, TEV protease recognition site, multiple cloning site, *trc* promoter, *lacI*^{q} gene, *Amp*^{R}, pBR322 and bacteriophage F1 origin of replication. Maintained in *E.coli* DH5α.	Life Technologies, USA
pGEMT-XVGrp94	*Lac*^{z}, *Amp*^{R}, *XVGrp94*. Maintained in *E. coli* JM109.	This work
pGEMT-XVGrp94(ORF)	*Lac*^{z}, *Amp*^{R}, *XVGrp94* ORF. Maintained in *E. coli* JM109.	This work
pProEX HTa-XVGrp94	6x histidine affinity tag, TEV protease recognition site, *XVGrp94* ORF , *trc* promoter, *lacI*^{q} gene, *Amp*^{R}, pBR322 and bacteriophage F1 origin of replication. Maintained in *E. coli* DH5α.	This work

4.2.2. Construction of plasmids

For the construction of pGEMT-XVGrp94(ORF), oligonucleotide primers were designed to amplify a region of the XVGrp94 gene encompassing only the ORF from the first ATG to the stop codon. pGEMT-XVGrp94 (C.2.1) was used as template and the forward primer, Hsp90atg2 (C.1.5) and reverse primer, Hsp90Rev3`A (C.1.2), were used. The reaction was performed in a volume of 50 µl using Long Template High Fidelty Taq Polymerase (Roche, Germany) according to the manufacturer's specifications. The resultant 2.45-kb band was gel purified (A.1.6.1) and ligated into pGEMT-Easy according to the manufacturer's protocol. The ligation mix was used to transform *E. coli* JM109 using the protocol described in appendix A (A.1.5.1) and transformants were selected on LA supplemented with 100 mg/ml ampicillin (B.14.2), 0.5 mM IPTG (B.14.8) and 80 µg/ml X-Gal (B.14.19). White colonies were screened by colony PCR (A.1.6.4.3) with the M13 Forward (C.1.7) and Reverse (C.1.8) primers. The following protocol was used: (1) 94°C for 5 minutes (2) 94°C for 30 seconds (3) 55°C for 30 seconds (4) 72°C for 3 minutes and (5) 72°C for 7 minutes. Steps 2-4 were repeated 25 times. Products were electrophoresed on a 0.8% TBE gel. A recombinant plasmid containing the *Hsp90* insert was named pGEMT-XVGrp94(ORF) (C.2.1a) and harvested using the High Pure plasmid mini-preparation kit (Roche, Germany) according to the manufacturer's recommendations and the insert was sequenced using the M13 Forward (C.1.7) and Reverse primers (C.1.8). For subsequent cloning steps pGEMT-XVGrp94(ORF) was also harvested using the large-scale isolation of plasmid DNA protocol described in A.1.1.3.

For the construction of pProEX HTa-XVGrp94 (C.2.2), *Hsp90* was excised from pGEMT-XVGrp94(ORF) (A.1.6.5.2) using *Not*I. pProEX HTa was also digested with *Not*I and the linear plasmid was dephosphorylated (A.1.6.7). The linear plasmid and the *Hsp90* insert were gel-purified and the ligation was conducted at a ratio of *Hsp90* insert to plasmid of 2:1. The ligation mix was used to transform *E. coli* DH5α and transformants were selected on LA containing 100 mg/ml ampicillin. Putative transformants were screened for the presence and correct orientation of Hsp90 by colony PCR. The reaction was performed using the M13 Reverse primer as a forward primer and the reverse primer, Rev5`C (C.1.10). A putative recombinant pProEX HTa clone containing the *Hsp90* insert in the correct orientation was selected and harvested using the small-scale isolation of *E. coli* plasmid explained in A.1.1.2 and confirmed using restriction endonuclease digestion (A.1.6.5.2). A plasmid containing the insert in the correct orientation was harvested using the High Pure Plasmid Preparation Kit (Roche, Germany) and the junction between the 6x histidine tag and the 5` region of the insert was sequenced using the M13 Reverse primer.

4.2.3. Small scale induction of pProEX HTa-XVGrp94

The optimal sampling time for maximal protein yield was determined using a small scale induction. A single colony was streaked onto LA containing 100mg/ml ampicillin and grown at 37°C overnight. A single colony from this plate was used to inoculate 5 ml LB containing 100 mg/ml ampicillin and the culture was grown at 37°C overnight, with shaking. A 10 ml LB culture (containing 100 mg/ml ampicillin) was inoculated with 0.1 ml of the overnight culture and incubated at 37°C, with shaking. The culture was allowed to grow until the OD_{590} reached 0.5, at which point 1 ml of the culture was removed. This was centrifuged at 12 000 rpm for 2 minutes in a microcentrifuge, the supernatant was discarded and the pellet resuspended in 100 µl cell disruption buffer (B.12.1) and stored at –20°C. This was the uninduced sample. To the remaining culture IPTG was added to a final concentration of 0.6 mM and the culture was then incubated at 30°C, with shaking. After induction, 1 ml aliquots were removed from the culture at hourly intervals for three hours. Each aliquot was centrifuged at 12 000 rpm in a microcentrifuge, the supernatant discarded and the pellet resuspended in 100 µl cell disruption buffer and stored at –20°C. 10 µl aliquots of the uninduced and induced samples were mixed with an equal volume of 2x SDS sample buffer (B.8.7) and analysed by SDS PAGE (A.2.2). The gels were stained with Coomassie blue (A.2.4).

4.2.4. Large scale induction of pProEX HTa-XVGrp94

A 10 ml LB culture, containing 100 mg/ml ampicillin was inoculated with a single fresh colony and incubated overnight as for the small scale induction. Five flasks, each containing 100 ml LB with 100 mg/ml ampicillin, were each inoculated with 0.1 ml of the overnight culture. These were incubated at 37°C, with shaking, until they reached an OD_{590} of 0.5. At this point an uninduced 100 ml sample was removed, centrifuged at 5000 rpm at 4°C for 10 minutes and the supernatant removed. The weight of the pellet was determined, frozen and stored at –70°C. To the other four 100 ml cultures IPTG was added to a final concentration of 0.6mM. The cultures were then allowed to incubate at 30°C, with shaking for the optimal three hours. At the end of the induction period the remainder of the cells were harvested and stored in the same manner as for the uninduced sample.

The frozen pellets for both the uninduced and induced samples, harvested above, were resuspended in 4 volumes of lysis buffer 1 (B.13.1) and 8 µl 50 mM PMSF (B.14.12) and 80 µl of an 10 mg/ml stock of lysozyme (B.13.3) were added per 1 g of cells. The mixture was aliquoted into sterile 1.5 ml Eppendorf tubes and left on ice in the fumehood for 30 minutes.

Triton-X was added to a final concentration of 1%, the mixture mixed and incubated at 37°C until it became viscous (approximately 30 minutes). Aliquots (5 µl) DNAseI (1 mg/ml; B.13.4) and RNAse A (10 mg/ml; B.14.13) were added per 1 ml lysis buffer 1 and this was incubated at room temperature for 30 minutes. The tubes were centrifuged at 12 000 rpm in a microcentrifuge at 4°C for 15 minutes. The supernatant was removed carefully, placed in a fresh sterile 1.5 ml Eppendorf tube and stored at –70°C in 1 ml aliquots.

The remaining pellets were resuspended in 9 volumes of cold lysis buffer 2 (B.13.2) and the tubes were incubated at room temperature for 5 minutes. This was followed by centrifugation at 12 000 rpm for 15 minutes at 4°C in a microcentrifuge. The supernatant was removed and placed in a fresh sterile 1.5 ml Eppendorf tube and the remaining pellet resuspended in 100 µl 1X PBS (B.13.5). These were stored at –70°C. Aliquots (10 µl) of each sample were mixed with an equal volume of 2x SDS sample buffer (B.8.7), boiled for 5 minutes and analysed by SDS-PAGE . The gels were stained with Coomassie blue.

Protein purification was attempted with Ni-NTA resin (Qiagen, Germany) in the form of a slurry in a 1.5 ml Eppendorf tube. The Ni-NTA resin was equilibrated using: 400 µl of Ni-NTA slurry for each 1 ml protein extract. The slurry was centrifuged at 12 000 rpm, in a microcentrifuge for 30 seconds at 4°C. The supernatant was removed and the pellet washed twice in Buffer A (B.13.6) by adding half the volume of the original slurry, mixing thoroughly, incubating on ice for 5 minutes and finally centrifuging at 12 000 rpm in a microcentrifuge at 4°C. Protein extract (1 ml) was added to the 400 µl slurry and mixed for 20 minutes at 4°C on a shaker. The mixture was centrifuged at 12 000 rpm in a microcentrifuge at 4°C for 1 minute and the supernatant, containing the proteins that did not bind to the resin, transferred to a sterile 1.5 ml Eppendorf tube. The resin was then washed with 1 ml Buffer A for 5 minutes at 4°C. The tube was centrifuged at 12 000 rpm at 4°C and the supernatant was transferred to a fresh 1.5 ml Eppendorf tube. This was repeated once for every 100 µl of slurry (i.e. a 400 µl column was washed four times). To elute the protein 100 µl of Buffer C (B.13.7) was added per 100 µl slurry and mixed at 4°C for 5 minutes. The mixture was centrifuged at 12 000 rpm in a microcentrifuge at 4°C for 1 minute and the supernatant transferred to a fresh 1.5 ml Eppendorf tube. These steps were repeated three times to elute all the protein from the column. All samples were stored at –70°C. For analysis by SDS-PAGE, 25 µl of each sample was mixed with an equal volume of 2x SDS sample buffer and boiled for 5 minutes before electrophoresis. The gels were stained with Coomassie blue.

4.3. RESULTS AND DISCUSSION

4.3.1. Construction of pProEX HTa-XVGrp94

The original *XVGrp94* transcript cloned into pGEMT-Easy to generate pGEMT-XVGrp94 (C.2.1) was found to contain a TAG stop codon (Figure 2.4, nucleotides 96 to 98) before the first ATG (nucleotides 114 to 116). This would prevent the expression of the recombinant protein in the pProEX™ HT protein expression system as translation would terminate between the his tag and *XVGrp94* coding region. Although *XVGrp94* has its own potential start codon, there is no ribosomal binding site before this start codon. Also purification would be complicated because if the protein was successfully translated it would not be fused to a his tag. Therefore the 2.45-kb ORF (from positions 114 to 2559) of the *XVGrp94* gene, was PCR-amplified (Figure 4.1), cloned into pGEMT-Easy and screened by PCR (Figure 4.2). The resulting construct was named pGEMT-XVGrp94(ORF) (C.2.1a).

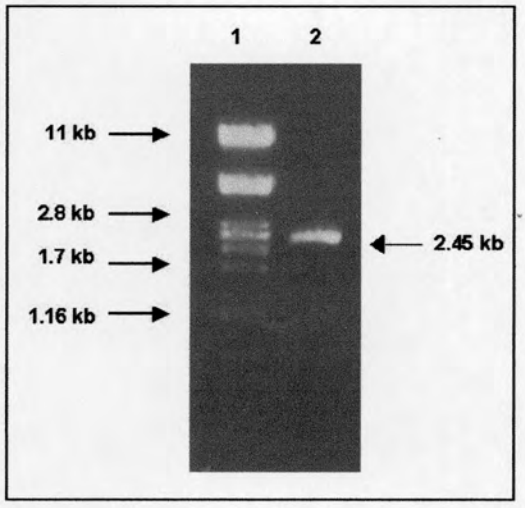


Figure 4.1: PCR-amplified *XVGrp94* ORF. Lane 1, λ *Pst* I DNA markers; lane 2, 2.45-kb PCR product. Arrows on the left hand side indicate molecular sizes determined from the λ *Pst* I markers.

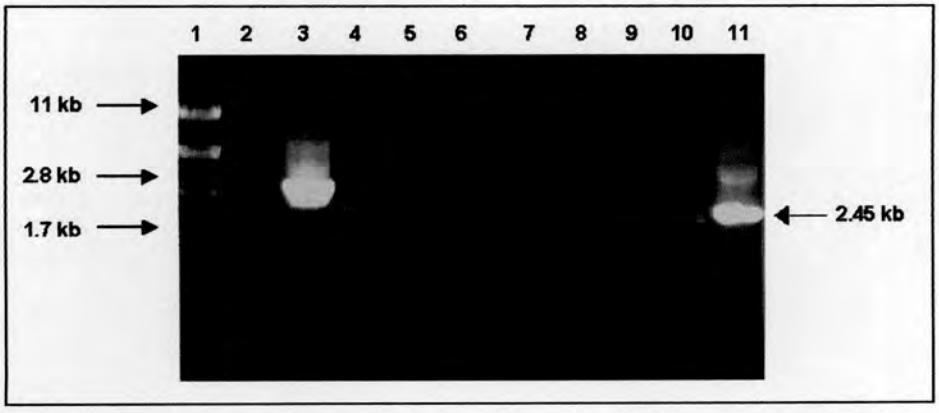


Figure 4.2: Products of the PCR screening for pGEMT-Easy containing the *XVGrp94* ORF. Lanes 1, λ *Pst* I DNA markers; 2 to 11, PCR-products from putative transformants. Lanes 3 and 11 represent colonies containing the *XVGrp94* ORF cloned into pGEMT-Easy. Arrows on the left hand side indicate molecular sizes determined from the λ*Pst* I markers.

As the pProEX HT™ series of expression vectors are available with the 6X histidine tag in three different reading frames relative to the multiple cloning site, the appropriate vector has to be chosen to ensure expression of the gene of interest as a fusion with the histidine tag. The sequence of the pProEX HT vectors as well as the *Hsp90* cDNA were analysed and it was determined that pProEX HTa would be the appropriate vector. The 2.45-kb *XVGrp94* cDNA was excised from pGEMT-XVGrp94(ORF) with *Not*I. This enzyme was chosen as it cuts within the multiple cloning site of the pProEX HTa vector and on both sides of the insert cloned into pGEMT-Easy, but not within the insert itself. The cDNA was then cloned into the *Not*I site of pProEX HTa, transformed into *E. coli* DH5α, and putative recombinant plasmids were screened for the presence and correct orientation of the *XVGrp94* insert by PCR. A set of primers was chosen that would yield a 1.4-kb product if the insert was in the correct orientation (Figure 4.3).

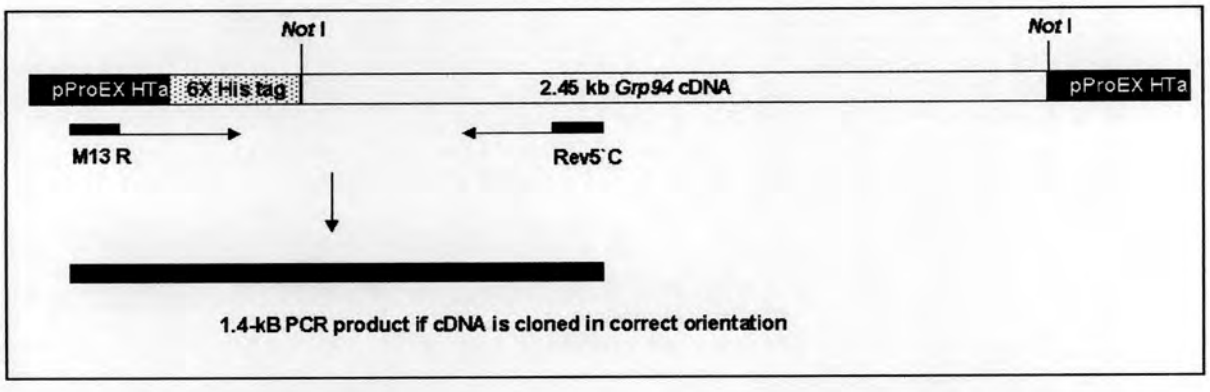


Figure 4.3: A representation of the method employed to screen recombinant pProEX HTa clones containing the *XVGrp94* ORF in the correct orientation. The M13 Reverse primer and a gene specific reverse primer were used.

Four plasmids containing the insert in the correct orientation were found (Figure 4.4) and confirmed by restriction endonuclease digestion (Figure 4.5). All four were digested with *Not*I, which was expected to release a 2.45-kb band from pProEX HTa-XVGrp94. These can be seen in lanes 6, 8 and 9. The clone in lane 7 is thought to be vector containing the insert that has lost one of the *Not*I sites. The clone represented by lane 6 was chosen for further manipulation and named pProEX HTa-XVGrp94 (C.2.2). The junction between the 6X histidine tag and the 5` end of the gene was sequenced to confirm that there had been no frame shifts in this region during cloning as this could result in loss of expression of the recombinant protein (Figure 4.6). From this it can be seen that the *Hsp90* insert is in frame with the multiple cloning site which is in fame with the histidine tag.

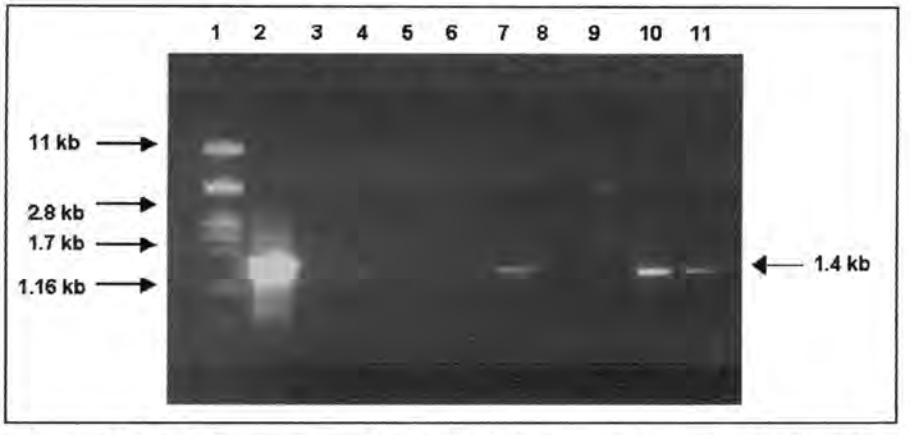


Figure 4.4: Products of the PCR-screening for pGEMT-Easy containing the *XVGrp94* ORF. Lanes 1, λ *Pst* I DNA markers; 2, positive control (pGEMT-XVGrp94); 3 to 11, PCR-products from putative transformants. Lanes 4, 7, 10 and 11 represent colonies containing *XVGrp94* ORF cloned into pProEX HTa in the correct orientation. Arrows on the left hand side indicate molecular sizes determined from theλ *Pst* I markers.

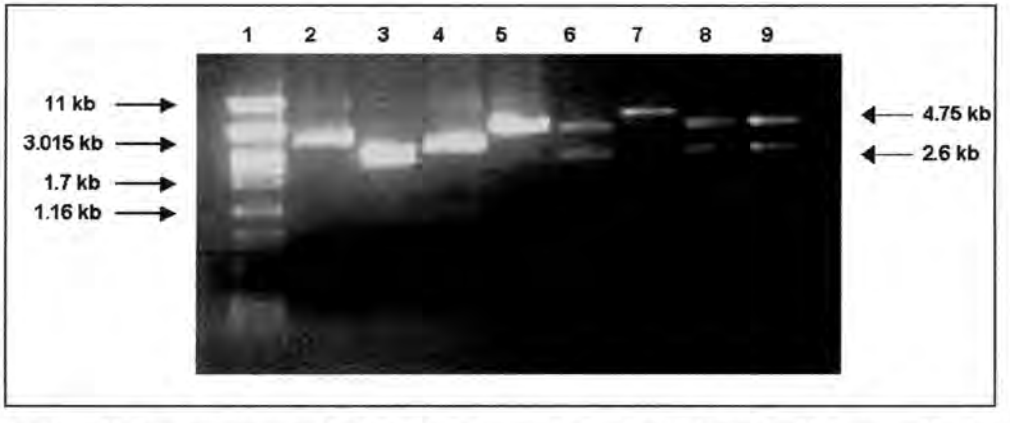


Figure 4.5: Restriction enzyme analysis of the clones shown by PCR analysis to contain the *XVGrp94* ORF in the correct orientation. Lanes 1, λ *Pst* I DNA markers; 2, pGEMT-XVGrp94(ORF) uncut; 3, pGEMT-XVGrp94(ORF) digested with *Not*I; 4, pProEX HTa uncut; 5, pProEX HTa digested with *Not*I; 6 to 9, putative transformants digested with *Not*I. Arrows on the left hand side indicate molecular sizes determined from the λ *Pst* I markers.

ATG TCG TAC TAC CAT CAC CAT CAC CAT CAC GAT TAC GAT ATC CCA ACG ACC

GAA AAC CTG TAT TTT CAG GGC GCC ATG GAT CCG GAA TTC AAA GGC CTA CGT

| *Not* I

CGA CGA GCT CAA CTA GTG CGG CCG CGG GAA TTC GAT T**CG ATG AGG AAT TGG**

TCG ATC CCC TCC TGC GCT CGT CCT CCT ACT TCT GAT TTC ACT CTC CGC AAT

CCC TGA TGG AGG CCG TAA GCT GCA CGC GAA CGC CGA GGA GAG CCG CGA

TGC CGA CGA GCT TGT GGA

Figure 4.6: Nucleotide sequence of *XVGrp94*(ORF) cloned into the *Not*I site of pProEX HTa. The sequence begins at the beginning of the multiple cloning site of the vector. The *XVGrp94* sequence is in-frame with the multiple cloning site and the 6X histidine tag (sequence not shown). *XVGrp94* sequence is in bold, the start codon is underlined. pGEMT-Easy sequence is underlined and the remaining sequence is pProEX HTa.

4.3.2. Small scale induction of pProEX HTa-XVGrp94

A small scale induction of the recombinant protein was conducted using *E. coli* DH5α cells transformed with the pProEX HTa-XVGrp94 vector (Figure 4.7). This was to verify that the protein was correctly expressed as well as to determine the optimum interval between induction and harvesting. A 94-kDa band, which was very faint in the uninduced sample was seen to increase in intensity over the three-hour duration of the induction. Although the protein encoded by the *Hsp90* cDNA, as cloned into the pProEX HTa vector, was originally deduced to be 92.83 kDa, the size discrepancy can be explained by the presence of the histidine tag, the spacer region, the TEV protease cleavage site as well as the residues before the *Not* I restriction site (C.2.2). The small scale induction showed that three hours was adequate as maximal protein production was observed after one hour with little significant increase up to three hours. It was decided to conduct the large scale induction over three hours.

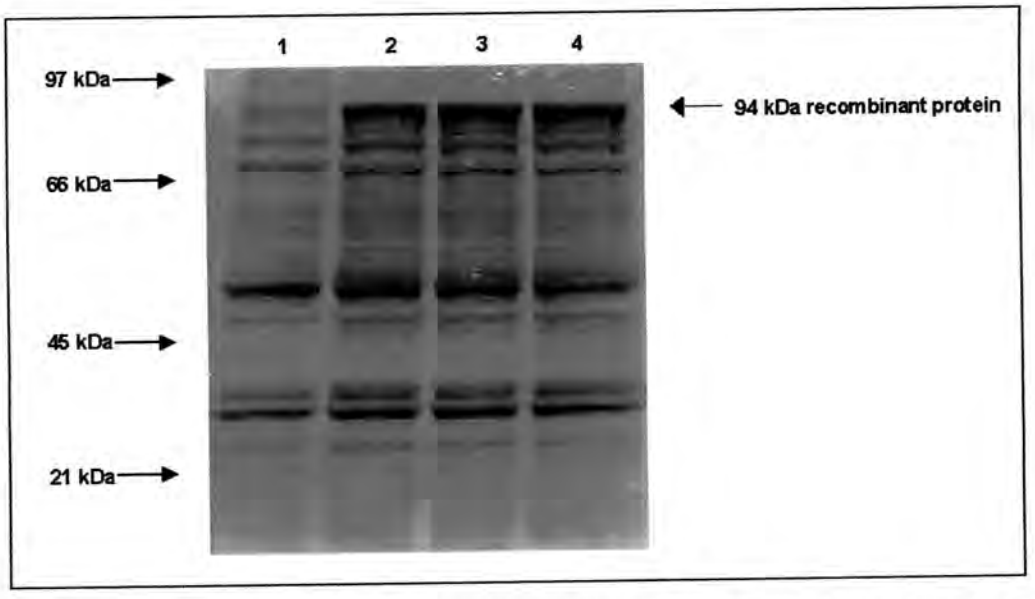


Figure 4.7: Polyacrylamide gel of the small scale induction. Lanes 1, uninduced sample; 2, 1 hour induction; 3, two hours induction; 4, three hour induction. Arrows on the left hand side indicate molecular sizes determined from the molecular weight markers.

4.3.3. Large scale induction of pProEX HTa-XVGrp94

A culture of *E. coli* DH5α transformed with pProEX HTa-XVGrp94 was induced with IPTG. The soluble proteins (in the supernatant) were extracted, the pellets washed to release proteins from inclusion bodies and resuspended in PBS and the products run on a 10% polyacrylamide gel (Figure 4.8). Lane 1 contains the soluble protein extract of the induced sample and it can be seen that there is a larger amount of the 94-kDa protein present

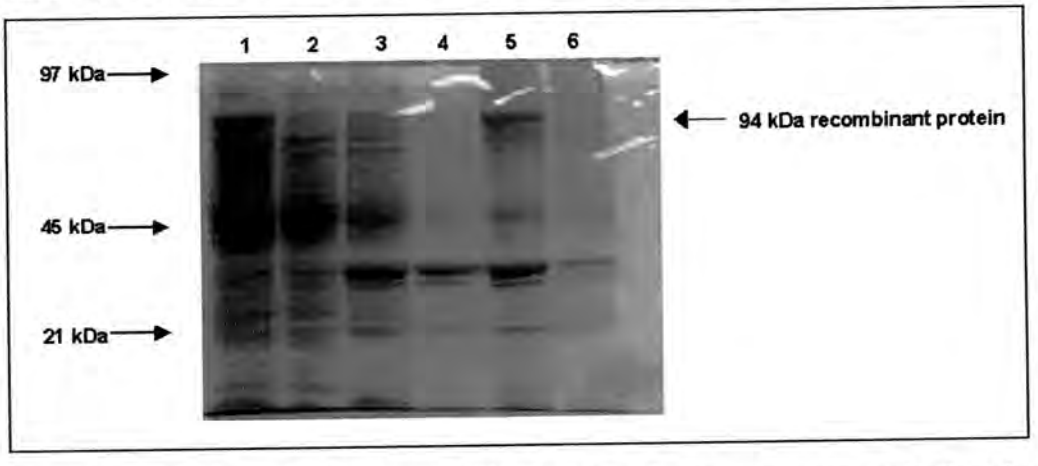


Figure 4.8: Polyacrylamide gel of the large scale induction. Lanes 1 supernatant (induced sample); 2, supernatant (uninduced sample); 3, pellet wash (induced sample); 4, pellet wash (uninduced sample); 5, pellet resuspended in PBS (induced sample); 6 pellet resuspended in PBS (uinduced sample). Arrows on the left hand side indicate molecular sizes determined from the molecular weight markers.

when compared to the uninduced sample (lane 2). The pellets were washed to release proteins from inclusion bodies and lanes 3 and 4 contain proteins from the induced and

uninduced samples, respectively. There is evidence of the release of small amounts of the 94-kDa protein from inclusion bodies. Lanes 5 and 6 contain proteins from the pellet redissolved in PBS. A significant amount of the 94-kDa protein was observed in the pellet fraction of the induced sample (lane 5) with no evidence of the 94-kDa protein in the uninduced sample. Unfortunately, the protein concentrations are unequal as it was not possible to quantitate them accurately due to the fact that the extraction buffer contained β-mercaptoethanol which interferes with the Bradford assay for quantification of proteins. However, it can still be seen that there is a significant amount of the 94-kDa protein in the pellet fraction (lane 5) and very little is released by washing of the inclusion bodies. This would suggest that the recombinant protein is mostly insoluble.

The insoluble fraction was used for the attempted purification of the recombinant protein (Figure 4.9). The insoluble protein extract was added to the resin, allowed to bind and the supernatant removed, this contained proteins that did not bind to the resin (lane 1). There was evidence of the 94-kDa protein eluting with these unbound proteins. The resin was then washed twice to remove other contaminating proteins (lanes 2 and 3). The washes contained low levels of imidazole which binds to the resin and displaces proteins (Janknecht et al., 1991), and is thus able to selectively elute contaminants that bind less strongly to the resin. Again the 94-kDa protein was seen to elute from the column and was completely lost by the second wash (lane 3). Proteins that had bound to the resin were then eluted with wash buffer containing a higher concentration of imidazole and there was no evidence of the presence of the recombinant protein, thus indicating that the protein was unable to bind to the resin (lane 4).

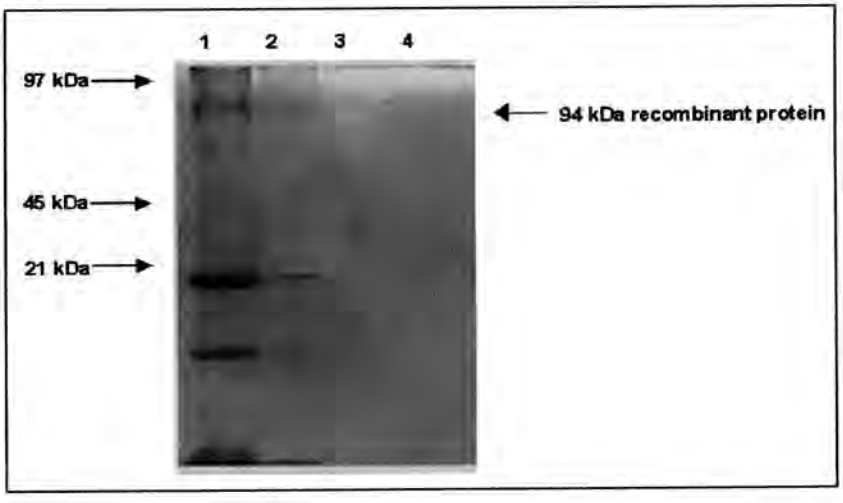


Figure 4.9: Polyacrylamide gel of the protein purification. Lanes 1, non-bound protein; 2, wash 1; 3, wash 2; 4, eluted proteins. Arrows on the left hand side indicate molecular sizes determined from the molecular weight markers.

It is recommended that insoluble proteins, or proteins located in inclusion bodies, should be solubilised by denaturation before purification (QIAexpress manual, Qiagen). This was not attempted as the purpose of purifying this protein was to conduct functional studies. Although it is possible to renature the protein while it is attached to the resin it is not possible to determine if the protein has renatured properly. In some cases the 6X His tag is hidden by the tertiary structure of the native protein, so that soluble as well as insoluble proteins require some level of denaturation before they can be purified on Ni-NTA. If denaturation of the protein is undesirable, the problem may be solved by moving the 6X his tag to the opposite terminus of the protein (QiaExpress manual, Qiagen). This is a possibility for the recombinant XVGrp94 as Hsp90s have been shown to possess a high degree of tertiary structure at the N-terminus (Prodromou *et al.*, 1997a, Stebbins *et al.*, 1997). The tag may therefore be hidden at the N-terminal domain of the protein and expression at the C-terminal domain could alleviate this problem. However, it must be kept in mind that the C-terminal domain is the site of dimerisation of the native protein (Nemoto *et al.*, 1995, Meng *et al.*, 1996; Nemoto *et al.*, 1997; Nemoto and Sato, 1998), and the tag may block this domain thus hindering dimerisation which is essential for Hsp90 function (Minami *et al.*, 1994). Removal of the tag will, however, ensure that the C-terminal domain is not blocked.

In vivo alternatives are available for conducting functional studies, one of which is a yeast-expression system. In this case the gene is cloned into a yeast expression vector which is transformed into a yeast strain deficient in Hsp90. The growth of these mutants can be monitored at normal as well as elevated temperatures to determine whether the introduced gene can complement a null mutant for the gene. Another system available is an *A. thaliana* cell suspension which can be used for analysis of chaperone activity of proteins *in vivo* (Forreiter *et al.*, 1997). This system involves a stably transformed cell line expressing high levels of firefly luciferase, and this can be used to determine the thermal denaturation and renaturation of the luciferase enzyme and the protective role of different chaperones.

CHAPTER FIVE

GENERAL CONCLUSIONS

Drought is responsible for large crop losses every year. In order to produce crops better able to withstand environmentally imposed stresses, the mechanisms employed by plants to avoid or tolerate such stresses, need to be understood. Resurrection plants, such as *X. viscosa*, offer ideal biochemical and genetic models to study these processes. A *X. viscosa* cDNA library was screened in order to isolate genes that are upregulated during conditions of dehydration. A cDNA encoding a putative Hsp90 was isolated and sequenced. The sequence contains the Hsp90 family signature (NKDIFL) as well as a putative ER localisation signal (eukaryotic secretion signal) at the amino terminus of the protein, and the ER-retention signal at the C-terminal end, suggesting that this gene represents the *Hsp90* ER homologue, often referred to as Grp94 or endoplasmin. The protein also contains many conserved residues in the N-terminal region suggesting that it contains an ATP-binding domain and ATPase activity similar to that observed for DNA gyrase B, MutL, EnvZ and CheA histidine kinases and the Hsp90 proteins from yeast and human (Wigley *et al.*, 1991; Ban *et al.,* 1999; Bilwes *et al.,* 1999; Prodromou *et al.*, 1997b; Stebbins, 1997). These proteins all belong to a newly emerging family of ATPases that function as a molecular clamp. ATP binding promotes the association of the N-termini of a protein dimer, hinged at the C-terminal domains. This results in the formation of a molecular clamp, the opening and closing of which is directly linked to the ATPase cycle (Ban *et al.,* 1999; Kampranis *et al.,* 1999).

Southern blot analysis confirmed the presence of the gene in the *X. viscosa* genome, possibly as a single copy. Analysis of the Southern blot was difficult and possibly confounded by the presence of introns within the chromosomal copy or the presence of closely related homologues. Retrieval of the gene from the chromosome would reveal the presence of introns and make interpretation of the Southern blot more conclusive. This sequence would also facilitate the analysis of the promoter region of *XVGrp94* thereby revealing possible mechanisms of regulation.

Western blot analysis revealed that XVGrp94 levels increased dramatically in response to heat and dehydration. This would indicate that this protein has a protective role during conditions of such stress. As XVGrp94 is member of the Hsp90 family it is thought that it may behave as a molecular chaperone. It can therefore be suggested that XVGrp94 could be involved in protecting proteins during stressful conditions that result in protein denaturation and/or damage. In order to confirm this, the function of XVGrp94 needs to be determined. The function of a protein can be inferred from comparisons with other sequences of known function, however, this is not a substitute for functional analysis. There are a number of methods available to determine the function of a protein. *In vitro* studies entail the purification of the recombinant protein in order to perform functional studies with known substrate proteins, whilst *in vivo* methods involve complementation of null-mutants in order to assess whether the protein is able to fulfill the role of the protein whose gene has been knocked out. In this work the protein encoded by the XVGrp94 cDNA was expressed in *E. coli*, however attempts to purify it were unsuccessful. Alternative methods of purification, such as the addition of detergents or the use of expression vectors that result in a fusion protein with the tag on the C-terminal end may alleviate the problems associated with purification. If it is not possible to purify the protein to the levels required for *in vitro* functional analysis, expression of the XVGrp94 protein in knock-out mutants, such as yeast deficient in Hsp90, may allow us to determine XVGrp94 function *in vivo.*

This work has indicated that the putative ER-located Hsp90, XVgrp94, may play a role in the survival mechanism employed by *X. viscosa* to survive conditions of stress. As Hsp90 is widely distributed in all organisms the mere presence of this gene is not likely to account for the increased drought tolerance of *X. viscosa* relative to other plants. In combination with the other mechanisms employed by *X. viscosa*, differences in the degree, timing and spatial expression patterns of XVGrp94 may contribute to the drought tolerance exhibited by the plant. Bearing this in mind, it may be of relevance to study factors involved in the regulation of XVGrp94 expression under various conditions. Also, a means to determine the importance of this protein during dehydration is to overexpress it in a drought sensitive plant and assess its effects during stress.

APPENDIX A

PROTOCOLS

A.1. General protocols for nucleic acids.

A.1.1. DNA extractions

A.1.1.1. Genomic DNA extraction

Approximately 0.5 g of *X .viscosa* leaf material was ground to a fine powder in a mortar and pestle with liquid nitrogen and resuspended in 15 ml extraction buffer (B.1.1) in SS34 centrifuge tubes. To this was added 1 ml 20% SDS (B.14.15) and the mixture was mixed thoroughly by vortexing for 5 minutes after which it was incubated at 65°C for 10 minutes. A 5 ml volume of 5 M potassium acetate (B.1.2) was added, mixed thoroughly and then incubated on ice for 20 minutes. Centrifugation at 12 000 rpm for 20 minutes allowed for separation of the cellular debris from the nucleic acid and the supernatant was decanted into a clean SS34 centrifugation tube. 10 ml of isopropanol was added followed by incubation at –20°C for 30 minutes. The tube was centrifuged at 10 000 rpm for 15 minutes, the supernatant was removed and the pellet was air dried for 5 minutes. The pellet was then resuspended in 0.7 ml of a Tris buffer (50 mM Tris, 10 mM EDTA pH8.0) and aliquoted into sterile 1.5 ml Eppendorf tubes, to which 10 µl of 10 mg/ml RNAse A (B.14.13) was added. The tubes were incubated at 37°C for 30 minutes. The samples were then extracted with an equal volume of phenol (B.14.11), followed by phenol/chloroform/isoamyl alcohol (B.14.3) and finally chloroform. 1/10 volume of 3 M sodium acetate (B.1.3) and 2.5 volumes absolute ethanol were added, mixed thoroughly and incubated at –20°C overnight. The tubes were centrifuged at 12 000 rpm in a microcentrifuge at 4°C for 20 minutes, after which the pellets were washed with cold 70% ethanol (B.14.6). The pellets were then air-dried for 5 minutes and resuspended in TE buffer (B.14.17).

A.1.1.2. Small-scale isolation of *E. coli* plasmid DNA (miniprep)

A 5ml culture was grown overnight in LB (B.15.1) containing the relevant antibiotic(s). A 1.5ml volume of the culture was centrifuged in a 1.5ml microcentrifuge tube at 12 000 rpm and resuspended in 200 µl of solution I (B.4.1). After incubation at room temperature for 5 minutes, 400 µl of solution II (B.4.2) was added, and the mixture was mixed thoroughly by vortexing and incubated on ice for 5 minutes. A 300 µl volume of ice-cold solution III (B.4.3) was added and the sample was again vortexed briefly before being incubated on ice for a further 5 minutes. The sample was then centrifuged for 10 minutes at 4°C at 12 000 rpm to

remove cellular debris and denatured chromosomal DNA. The supernatant was transferred to a clean microcentrifuge tube and an equal volume if isopropanol was added. DNA was harvested by centrifuging at 12 000 rpm for 5 minutes, washed in 70% ethanol, dried, and resuspended in 20 µl of TE buffer (B.14.17).

A.1.1.3. Large-scale isolation of plasmid DNA from *E. coli* (maxiprep)

Plasmid DNA was isolated and purified using a Nucleobond plasmid DNA extraction kit (Boehringer Mannheim, Germany), following the manufacturer's instructions.

A.1.2. Agarose gel electrophoresis of nucleic acids

A.1.2.1. Agarose gel electrophoresis of DNA

DNA was separated according to size using agarose gels ranging in density between 0.8% and 1.0% w/v agarose, depending on the size of fragments to be separated. Agarose was mixed with 1xTBE (B.14.16). The mixture was heated to boiling point in a microwave oven, and then left to cool to approximately 50°C. Ethidium bromide (B.14.7) was added to a final concentration of 0.2 µg/ml, before the gel was poured and left to solidify.

Sample buffer (B.14.14) was mixed with the DNA samples before loading, to a final concentration of 1x. Gels were run in tanks containing 1xTBE at between 15V and 120V, depending on the size of the tank and the degree of separation required. DNA bands were visualized on a UV transilluminator (Protea Lab Services, Cape Town) at 254nm, or 310nm if the DNA was to be recovered. Photographs were taken using a Pharmacia Biotech Gel Display System. Phage λ DNA digested with *Pst*I (A.1.6.8) was run with the samples as a size standard. This was used to plot a standard curve of size vs. distance traveled to calculate sample DNA fragment sizes.

A.1.2.2. Agarose gel electrophoresis of RNA

RNA was separated according to size using a 1.2% agarose gel. Agarose was mixed with 1xTBE, which had been treated with 0.01% DEPC (B.14.4). The mixture was heated to boiling point in a microwave oven, and then left to cool to approximately 50°C. Ethidium bromide was added to a final concentration of 0.2 µg/ml, before the gel was poured and left to solidify. The gel tray and running tank were soaked in 10% sodium hypochlorite solution and wiped with methanol before use.

The RNA samples were denatured at 65°C for 5 minutes, followed by the addition of 2 µl

10X Mops (B.14.9), 3.1 µl formaldehyde, 10 µl formamide and 2 µl sample buffer. Gels were run in tanks containing 1xTBE at 100V for 1 hour. The RNA was visualized on a UV transilluminator (Protea Lab Services, Cape Town) at 254nm and photographs were taken using a Pharmacia Biotech Gel Display System. Phage λ DNA digested with *Pst*I was run with the samples as a size standard.

A.1.3. Determining concentration of nucleic acids

A.1.3.1. DNA concentration

The DNA concentration was determined spectrophotometrically using the relationship A_{260} = 1 for 50 µg/ml of double-stranded DNA.

A.1.3.2. RNA concentration

The RNA concentration was determined spectrophotometrically using the relationship A_{260} = 1 for 40 µg/ml of single-stranded RNA.

A.1.4. Nucleic acid transfer and hybridisation

A.1.4.1. Genomic DNA transfer

The top left hand corner of the gel was nicked, inverted and placed back on its tray. The gel together with its tray was placed in 500 ml of 0.25 N hydrochloric acid (B.2.1) for 10 minutes. The gel was rinsed in sterile dH_2O twice to remove excess hydrochloric acid and then placed in 500 ml denaturation solution (B.2.2) for 30 minutes. The gel was rinsed twice again and placed in 500 ml neutralization solution pH 8.0 (B.2.3) for 30 minutes. It was then placed on a wick soaked in transfer buffer (B.2.4) and a 12 cm x 15 cm Hybond XL membrane (Amersham, UK), presoaked in transfer buffer, was placed over the gel ensuring there were no airbubbles. The membrane was overlaid with three pieces of Whatman filter paper cut to the size of the membrane and paper toweling was stacked on top to a height of approximately 10 cm. A glass plate and a weight were placed on top of this. The transfer was set up in a tank containing transfer buffer and the wick was allowed to stay in contact with the buffer. Clingwrap was placed around the gel to prevent contact between the wick and the membrane or the paper toweling, thereby inhibiting short-circuiting of the transfer. The transfer was allowed to continue for 18 hours at room temperature.

A.1.4.2. Southern hybridization

Membranes were incubated, with shaking, at 65°C for 4 hours in prehybridisation buffer (B.3.1). The probe was then denatured at 100°C for 5 minutes, quick chilled on ice and then added to the prehybridisation solution. The membrane was hybridized with the probe at 65°C for 18 hours, with shaking. The hybridization solution was removed and the membrane washed with Wash Buffer A (B.3.4) for 10 minutes and Wash Buffer B (B.3.5) for 5 minutes, both at 65°C. The membrane was sealed in a plastic bag and placed in an X-ray cassette with Hyperfilm (Amersham, UK) X-Ray film and left to expose at –70°C for 5 days. The film was developed manually with Agfa developer and fixer.

A.1.4.3. RNA transfer

The top left hand corner of the gel was nicked and the gel was inverted and placed back in its tray. The gel was placed on a wick soaked in transfer buffer (B.2.5) and a 7 cm x 10 cm Hybond XL membrane (Amersham, UK), presoaked in transfer buffer, was placed over the gel, ensuring there were no airbubbles. The membrane was overlaid with three pieces of Whatman filter paper cut to the size of the membrane and paper toweling was stacked on top to a height of approximately 10 cm. A glass plate and a weight were placed on top of this. The transfer was set up in a tank containing transfer buffer and the wick was allowed to stay in contact with the buffer. Clingwrap was placed around the gel to prevent contact between the wick and the membrane or the paper toweling, thereby inhibiting short-circuiting of the transfer. The transfer was allowed to continue for 18 hours at room temperature. The tank used for the transfer was treated with 10% sodium hypochlorite solution and wiped with methanol before use.

A.1.4.4. Northern hybridization

Membranes were incubated in prehybridisation buffer (B.3.1) and hybridised with the denatured probe in the same manner as for the southern hybridisation. The hybridisation solution was removed and the membranes were washed twice in Wash Buffer A, for 5 minutes each, at 65°C. The membranes were sealed in plastic bags and placed in an X-ray cassette with Hyperfilm (Amersham, UK) X-Ray film and left to expose at –70°C for 10 days. The film was developed manually with Agfa developer and fixer.

A.1.5. Bacterial transformations

A.1.5.1. $RbCl_2$ competent cell transformation of *E. coli*

A 5 ml volume of Ψ broth (B.15.2) was inoculated with a single colony of bacteria to be transformed, and incubated overnight at 37°C with aeration. The entire 5ml was used to inoculate 200 ml of Ψ broth pre-warmed to 37°C. The culture was grown to mid-exponential phase (OD_{600} = 0.35), at which time the cells were transferred to a Beckman GSA tube and chilled on ice for 15 minutes. The cells were harvested by centrifugation at 2500 rpm for 15 minutes at 4°C. The cells were washed in a total volume of 21 ml of ice-cold TFB1 buffer (B.5.1), transferred to a Beckman SS34 tube and incubated on ice for 90 minutes. The cells were centrifuged again at 2500 rpm for 15 minutes at 4°C and gently resuspended in a total volume of 9 ml TFB2 (B.5.2). The suspension was divided into 100 µl aliquots, which were used for transformations or stored at -70°C.

For the actual transformation, cells were thawed on ice, after which 50-100 ng DNA was added to the 100 µl aliquot and the mixture incubated on ice for 20 minutes. The cells were heat-shocked at 37°C for 5 minutes, and returned to the ice for a further 2 minutes. A volume of 800 µl of LB (B.15.1), pre-warmed to 37°C, was added to the cells, which were incubated at 37°C for 45 minutes to allow expression of selectable marker genes. The cells were finally plated onto LA (B.15.1.1) with appropriate selection and incubated at 37°C until colonies were observed.

1.6. Nucleic acid manipulations

A.1.6.1. Gel purification

DNA was separated according to size by agarose gel electrophoresis (A.1.2.1). The relevant band was excised with a sterile blade and transferred into a sterile 1.5 ml Eppendorf tube. The DNA was purified from the gel slice using a High Pure PCR product Purification Kit (Roche, Germany) according to the manufacturers recommendations. The purified DNA was electrophoresed on a 0.8% TBE agarose gel to confirm that it was the correct size and the concentration was determined spectrophotometrically at a wavelength of 260 nm.

A.1.6.2. Random primed labeling

100 ng DNA in 9.0 µl dH_2O was denatured at 95°C for 10 minutes and then chilled on ice immediately. To this the following was added:

2.0 µl reaction mix

1.0 µl dATP

1.0 µl dTTP

1.0 µl dGTP

5.0 µl [^{32}P]dCTP

1.0 µl Klenow enzyme (1u/ µl).

The reaction was incubated at 37°C for 60 minutes.

A.1.6.3. Purification of radiolabelled probe

Spun-column procedure

A 1 ml syringe was plugged with approximately 0.1 ml glass wool and a column of Sephadex G-50 (B.6.1) equilibrated in STE buffer (B.6.2) was prepared in the syringe. 0.9 ml of Sephadex was added to the syringe, which was placed in a 10 ml Sterilin tube (Bibby Sterilin, England). The syringe, in the Sterilin tube was centrifuged at 1600 rpm for 1 minute. After centrifugation more Sephadex was added and the tube was centrifuged again. This was repeated until a 0.9 ml column had been prepared. 0.1 ml STE buffer was added to the column and it was centrifuged again at 1600 rpm. A sterile 1.5 ml Eppendorf tube (with the lid removed) was placed at the bottom of the Sterilin tube to catch the eluted probe.

10 µl tracking dye (B.6.3) and 40 µl STE buffer was added to the labeled DNA. The DNA was loaded onto the column and was centrifuged for 6 minutes at 1600 rpm to elute the labelled probe into the Eppendorf tube.

A.1.6.4. Standard PCR reactions

Unless otherwise stated, all PCR reactions were performed using Super-Therm DNA polymerase (SR products, Kent, UK). All PCR reactions were performed in Perkin-Elmer (PE) DNA Thermal cycler 9700 (Applied BioSystems).

A.1.6.4.1. Standard PCR reaction

dH_2O	x µl
Buffer (10 X)	5.0 µl
$MgCl_2$ (25 mM)	3.0 µl
dNTPs (2.5 mM)	4.0 µl
Forward primer (10 µM)	2.5 µl
Reverse primer (10 µM)	2.5 µl
Taq polymerase (5u/µl)	0.20 µl
DNA template (50 ng)	y µl
Total	50.0µl

A.1.6.4.2. Radioactive PCR-labeling

dH_2O	x µl
Buffer (10 X)	5.0 µl
$MgCl_2$ (25 mM)	3.0 µl
dNTPs (0.5 mM) 1:1:1	3.0 µl
[^{32}P]dCTP	5.0 µl
Forward primer (10 µM)	2.5 µl
Reverse primer (10 µM)	2.5 µl
Taq polymerase (5u/µl)	0.20 µl
DNA template (50 ng)	y µl
Total	50.0µl

The cycles used were as follows: (1) 94°C for 3 minutes (2) 94°C for 30 seconds (3) 54°C for 1 minute (4) 72°C for 10 minutes (5) 72°C for 10 minutes. Steps 2 to 4 were repeated 15 times.

A.1.6.4.3. Colony PCR

dH_2O	16.3 µl
Buffer (10 X)	2.5 µl
$MgCl_2$ (25 mM)	1.5 µl
dNTPs (2.5 mM)	2.0 µl
Forward primer (10 µM)	1.25 µl
Reverse primer (10 µM)	1.25 µl
Taq polymerase (5u/µl)	0.20 µl
Total	25.0 µl

The PCR mix was prepared and a single colony was picked, with a sterile toothpick, and transferred to the PCR mix. This was then mixed well by vortexing and centrifuged for 30 seconds at 12 000 rpm in a microcentrifuge.

A.1.6.5. DNA restriction digests

A.1.6.5.1. Genomic DNA restriction digest

dH_2O	x µl
Restriction enzyme buffer (10 X)*	5.0 µl
Enzyme	5.0 µl
Genomic DNA (10 µg)	y µl
Total	50.0 µl

Incubate at 37°C overnight, unless specified otherwise.

*Restriction buffer as specified by the manufacturer.

A.1.6.5.2. Plasmid DNA restriction digest

dH_2O	x µl
10X Restriction enzyme Buffer	2.0 µl
Restriction enzyme (10u/µl)	2.0 µl
Plasmid DNA (1 µg)	y µl
Total	20.0 µl

Incubate at 37°C (unless specified otherwise) for 2-3 hours.

A.1.6.6. Ligation reaction

dH_2O	x µl
10X Ligase Buffer	2.0 µl
Plasmid DNA	y µl
Insert DNA	z µl
Ligase (1u/µl)	2.0 µl
Total	20.0 µl

Incubate at 14°C overnight.

A.1.6.7. Dephosphorylation of plasmid DNA with CIP (calf intestinal phosphatase)

This reaction was performed immediately after completion of digestion in the same Eppendorf tube. The restriction enzyme was heat inactivated at 70°C for 10 minutes.

To the 20.0 µl digestion volume add the following:

5 µl CIP buffer (10x)

5 µl CIP enzyme (1 u/µl)

Bring the volume up to 50.0 µl with dH_2O

Incubate at 37°C for 15 minutes and then at 55°C for 45 minutes

Heat inactivate the CIP enzyme at 70°C for 15 minutes.

A.1.6.8. λ *Pst*I marker

dH_2O	73.0 µl
10X Buffer H	10.0 µl
λ phage DNA (0.45 µg/µl)	15.0 µl
*Pst*I restriction enzyme (1u/µl)	2.0 µl
Total	20.0 µl

Incubate at 37°C for 3 hours.

A.2. General protocols for proteins

A.2.1. Protein isolation from *X. viscosa* and *C.roseus.*

Leaf material was ground to a fine powder in a mortar and pestle in liquid nitrogen. The material was then transferred to a sterile 1.5 ml Eppendorf tube and dissolved in 400 µl Laemmli sample buffer (B.7.1). The samples were then mixed thoroughly for 5 minutes and then centrifuged at 12 000 rpm in a microcentrifuge. The supernatant was carefully removed and placed in a clean 1.5 ml Eppendorf tube and PMSF (B.14.4) was added to a final concentration of 1 mM. The protein samples were stored at –70°C.

A.2.2. SDS-PAGE

A Mighty Small™ gel apparatus was used (Hoeffer, San Fransisco) according to the manufacturers specifications. The apparatus allowed for the simultaneous preparation and electrophoresis of two gels. 10% resolving gels (B.8.5) were prepared and poured between the glass plates and overlayed with 1 ml isopropanol each. These were allowed to polymerise for 1 hour. Once the resolving gel had polymerised, the isopropanol was removed and the area above the gel was washed thoroughly to remove excess isopropanol. A 5% stacking gel (B.8.6) was prepared and poured slowly over the resolving gels, combs were inserted gently and the gels were again allowed to polymerise for 1 hour. Once polymerisation had occurred the combs were removed, the glass plates containing the gels were clamped to the running tank and 1X Running buffer (B.8.9) was added to the top and bottom tanks.

The protein samples, in Laemmli buffer, were thawed and 20 µl of each sample was aliquoted into a fresh 1.5 ml Eppendorf. 2 µl of 2X SDS sample buffer (B.8.7) was added to each tube and the samples were boiled for 5 minutes, after which they were cooled on ice. The samples were loaded into each well and two gels were loaded (i.e. each gel was run in duplicate). The gels were run at 100 V for 1- 2hours. Once the gels had run to completion, the power pack was switched off, the running buffer was poured off and the gels removed

from the running tank. The plates were removed and one gel was stained with Coomassie blue (A.2.4) and the other gel was used for western transfer (A.2.3). Molecular weight (MW) was determined by comparison to Bio-Rad low-MW standards (Bio-Rad, USA) which were run on the same gel.

A.2.3. Western transfer of proteins

Osmonics nitrocellulose membrane (pore size 0.45 μm) was cut to the size of the gel (8 cm x 6.5 cm) and was soaked in transfer buffer for 5 minutes. The stacking gel was removed from the resolving gel with a sterile blade and the top left hand corner of the gel was nicked. The gel was soaked in 1X Transfer Buffer (B.9.1) for 10 minutes with gentle shaking. A Mini VE western transfer apparatus (Hoefer, San Fransisco) was used. The apparatus was assembled and proteins transferred according to the manufacturers recommendations. The proteins were transferred at 100V for 1 hour in 1X Transfer Buffer. After completion of transfer the membrane was nicked at the corresponding corner and removed from the gel. The gel was stained with Coomassie blue (A.2.4). The membrane was placed in a container and stained with Ponceau S (B.10.2) for 5minutes at room temperature, with gentle shaking. The Ponceau S was removed and the membrane was destained in sterile dH_2O until the bands could be seen. Complete destaining was achieved by an additional 5 minute wash in sterile dH_2O.

A.2.4. Coomassie blue staining of polyacrylamide gels

The stacking gel was removed from the resolving gel with a sterile blade. The resolving gel was placed in a plastic tupperware container and covered with a layer of Coomassie blue stain (B.9.1). The gel was stained at room temperature with shaking for 30 minutes-1 hour. After this the stain was replaced with destain (B.9.2). The gel was left to destain on a shaker at room temperature overnight. Pieces of paper-toweling were added to the solution to aid the removal of the blue dye from the solution.

A.2.5. Immunodetection

The membrane was blocked in Blotto (B.11.2) for 1-2 hours at room temperature with gentle shaking. The membrane was then incubated with anti-Hsp90 diluted 1 in 1000 in Blotto overnight at 4°C, with gentle shaking. The membrane was washed three times for 5-10 minutes with Blotto at room temperature and placed in fresh Blotto containing a 1 in 5000 dilution of horse radish peroxidase conjugated goat anti-rabbit antibody (Sigma-Aldrich, UK). This was incubated at room temperature with gentle shaking for 4 hours. After this, the membrane was washed with 1X TBS (B.11.1) three times for 5-10 minutes at room

temperature and the bound antibody was detected using chemiluminescence. In a darkroom, the membrane was placed on a piece of Clingfilm protein side up. Solution 1 (B.11.3) and Solution 2 (B.11.4) were mixed and a thin layer of the solution was poured over the entire membrane surface and left for 1 minute. The membrane was transferred to a fresh piece of Clingfilm, the excess solution was drained and the membrane was covered with the Clingfilm. A piece of BioMax ML X-ray film (Kodak), cut slightly larger than the size of the membrane was placed on top and left to expose for 30 seconds, 1 minute and 2 minutes. The film was then developed manually, rinsed and allowed to air-dry. The X-ray film could then be overlayed on the duplicate Coomassie-stained gel to calculate the size of the bands detected.

APPENDIX B

BUFFERS, SOLUTIONS, MEDIA AND CALCULTIONS

B.1. Buffers for genomic DNA extraction

B.1.1. DNA extraction buffer

100 mM Tris pH 8.0

50 mM EDTA pH 8.0

500 mM NaCl

10 mM β-mercaptoethanol

B.1.2. 5 M potassium actetate

5 M potassium acetate

11.5 ml glacial acetic acid

Make up to 100 ml with dH_2O

Autoclave

B.1.3. 3 M sodium acetate

Make a 3 M sodium actetate solution in 400 ml dH_2O

pH the solution to 5.2 with glacial acetic acid

Make the volume up to 500 ml with dH_2O

Autoclave

B.2. Buffers for nucleic acid transfer

B.2.1. 0.25 N HCl

0.25 N HCl

Make up to 1 L with sterile dH_2O

B.2.2. Dentauration solution

1.5 M NaCl

0.5 M NaOH

Make up 1 L in dH_2O

Autoclave

B.2.3. Neutralisation solution (pH 8.0)

1.5 M NaCl

0.5 M Tris

Add 800 ml dH_2O

pH to 8.0 with HCl then make up to 1 L with dH_2O

Autoclave

B.2.4. DNA transfer buffer

0.4 M NaOH

Make 1 L in dH_2O

Autoclave

B.2.5. RNA transfer buffer

0.075 M NaOH

Made up in sterile dH_2O

Add DEPC to a final of 0.01% allow to stand at room temperature overnight

Autoclave

B.3. Buffers for Northern and Southern hybridisation

B.3.1. Prehybridisation/hybridisation buffer

1% BSA (B.3.2)

1 mM EDTA

0.5 M $NaHPO_4$ (B.3.3)

7% SDS

Make up to 100 ml with dH_2O

Make fresh before use

B.3.2. 10% BSA (bovine serum albumin)

Make up 10% BSA w/v in sterile dH_2O

Filter sterilize and store at –20°C

B.3.3. 1 M $NaHPO_4$ (pH 7.2)

1 M $Na_2HPO_4.7H_2O$

4 ml 85% H_3PO_4 (per litre)

Make up to 1 L with dH_2O

Autoclave

B.3.4. Wash buffer A

2 X SSC (B.3.6)

0.5% SDS

Make up to 100 ml with dH_2O

B.3.5. Wash buffer B

0.5 X SSC

0.1% SDS

Make up to 100 ml with dH_2O

B.3.6. 20 X SSC

3 M NaCl

0.3 M Sodium acetate

pH to 7.0 with NaOH

Make up to 1 L with dH_2O

Autoclave

B.4. Solutions for small-scale *E. coli* plasmid isolation

B.4.1. Solution I

0.25 M Tris-HCl pH 8.0

0.5 M Glucose

0.1 M EDTA pH 8.0

Autoclave glucose separately

Make up to 100 ml with dH_2O.

B.4.2. Solution II

0.2 N NaOH

1.0% SDS

Make up to 100 ml with dH_2O

Make fresh every week.

B.4.3. Solution III

3 M K-Acetete

2 M Acetic acid

Dissolve K-Acetate in 250 ml of dH_2O, adjust pH with glacial acetic acid and then make up to 500 ml with dH_2O .

B.5. Buffers for $RbCl_2$ competent cell transformation of *E. coli*

B.5.1. Transformation buffer 1 (TBF1)

100 mM $RbCl_2$

50 mM $MnCl_2.4H_2O$

30 mM Kac

10 mM

$CaCl_2$

15% glycerol

Adjust pH to 5.8 with glacial acetic acid, make volume up to 100 ml with dH_2O, filter sterilise.

B.5.2. Transformation buffer 2 (TFB2)

10 mM $RbCl_2$

75 mM $CaCl_2.2H_2O$

15% Glycerol

10 mM MOPS pH 7.0

Make up to 100ml with water, filter-sterilize.

B.6. Buffers and solutions for purification of radiolablled probes

B.6.1. Sephadex G-50

Slowly add 30 g sephadex G-50 (medium) to 250 ml STE buffer in a 500 ml beaker

Autoclave to make the beads swell

Cool to room temperature and store at 4°C

B.6.2. STE buffer

TE Buffer (B.14.17) containing 0.1 M NaCl

Autoclave

B.6.3. Spun column tracking dye

Dissolve Blue Dextran in 50 mM NaCl to a final concentration of 3.0%

Dissolve 1% Orange G (final concentration) in the above solution

Autoclave

B.7. Buffers for protein isolation

B.7.1. Laemmli sample buffer

0.625 M Tris-HCl pH 6.8

2% SDS

10% glycerol

5% β-mercaptoethanol

B.8. Buffers for SDS-PAGE

B.8.1. 4X Separating buffer

1.5 M Tris-Cl pH 8.8

Make up to 100 ml dH_2O

Autoclave

B.8.2. 4X Stacking buffer

0.5 M Tris-Cl pH 6.8

Make up to 100 ml with dH_2O

Autoclave

B.8.3. Ammonium persulphate

Make up 10% ammonium persulphate w/v in sterile dH_2O

Filter sterilise

Aliquot and store at –20°C

B.8.4. 10% SDS

Make up 10 % SDS w/v in sterile dH_2O

Heat to 80°C to dissolve and store at room temperature

B.8.5. 10% Polyacrylamide resolving gel

4 ml 40% acrylamide

4.5 ml 4 X separating buffer (B.8.1)

6.5 ml dH_2O

180 µl 10% SDS

180 µl 10% ammonium persulphate (B.8.3)

9 µl TEMED

B.8.6. 5% stacking gel

0.625 ml 40% acrylamide

2 ml 4 X stacking buffer (B.8.2)

4 ml dH_2O

70 µl 10% SDS

70 µl 10% ammonium sulphate

7 µl TEMED

B.8.7. 2X SDS sample buffer

125 mM Tris-HCl pH 6.8

4% SDS

20% glycerol

Make up to 10 ml with sterile dH_2O

B.8.8. 10X Standard buffer

250 mM Tris

2 M glycine

Make up to 1 L with dH_2O

Autoclave

Dilute to 1X with sterile dH_2O

B.8.9. 1X Running buffer

1X Standard buffer (B.8.8) with SDS to 0.1% final concentration

Prepare fresh just before use

B.9. Solutions for Coomassie blue staining of polyacrylamide gels

B.9.1. Coomasie stain

1 g Coomasie blue R250

45 ml dH_2O

45 ml methanol

10 ml acetic acid

B.9.2. Coomasie destain

300 ml methanol

100 ml acetic acid

600 ml dH_2O

B.10. Buffers for western transfer

B.10.1. 1X Transfer buffer

1X Standard buffer (B.8.8) with 200 ml/L methanol

Prepare fresh just before use

B.10.2. Ponceau S

Dissolve 0.5 g Ponceau S in 1 ml glacial acetic acid

Bring the volume up to 100 ml with dH_2O

Prepare just before use

B.11. Buffers and solutions for immunodetection

B.11.1. 10X TBS

200 mM Tris-HCl pH 7.4

1.5 M NaCl

Make up to 1L with dH_2O

Autoclave

Dilute to 1X with sterile dH_2O

B.11.2. Blotto

0.05% w/v dried, non-fat, milk powder

in 1X TBS (B.11.1)

Prepare fresh just before use

B.11.3. Solution 1

3 ml 100 mM Tris-HCl (pH 8.5)

13.3 µl Solution A (B.11.3.1)

1.66 µl hydrogen peroxide

B.11.3.1 Solution A

Dissolve 90 mM P-coumaric acid in DMSO.

Store at –20°C in a light safe container for no longer than 10 days.

B.11.4. Solution 2

3 ml 100 mM Tris-HCl (pH 8.0)

30 µl solution B (B.11.4.1)

B.11.4.1. Solution B

Dissolve 250 mM 3-Aminophalhydrazin in DMSO.

Store at –20°C in a light safe container for no longer than 10 days.

B.12. Buffers for small-scale induction of pProEX HTa-Hsp90

B.12.1. Cell disruption buffer

125 mM Tris-HCl pH 7.6

10% β-mercaptoethanol

10% Sucrose

0.001% Bromophenol blue

Make up to 10 ml with sterile dH_2O

B.13. Buffers for large-scale induction of pProEX HTa-Hsp90

B.13.1. Lysis buffer 1

50 mM Tris-HCl pH 8.5 (at 4°C)

5 mM β-mercaptoethanol

Make up to 10 ml with sterile dH_2O

B.13.2. Lysis buffer 2

50 mM Tris-HCl pH 8.0

5 mM β-mercaptoethanol

10 mM EDTA pH 8.0

0.5% triton-X 100

Make up to 10 ml with sterile dH_2O

B.13.3. Lysozyme (10 mg/ml)

Prepare a 10 mg/ml solution in sterile dH_2O

Filter sterilise, aliquot and store at –20°C

B.13.4. DNAseI (1 mg/ml)

Prepare a 1 mg/ml solution in sterile dH_2O

Dispense into aliquots and store at –20°C

B.13.5. 10X PBS (phosphate buffer)

15 mM NaCl

0.2 mM KCl

0.2 mM KH_2PO_4

Add 80 ml dH_2O adjust pH to 7.4 with HCl

Make the volume up to 100 ml with dH_2O

Autoclave

B.13.6. Buffer A

20 mM Tris-HCl pH 8.5 (at 4°C)

100 mM KCl

5 mM β-mercaptoethanol

10% glycerol

20 mM imidazole

Make up to 20 ml with sterile dH_2O and store at 4°C

B.13.7. Buffer C

20 mM Tris-HCl pH 8.5 (at 4°C)

100 mM KCl

5 mM β-mercaptoethanol

10% glycerol

100 mM imidazole

Make up to 20 ml with sterile dH_2O and store at 4°C

B.14. General buffers and solutions

B.14.1. Abscisic acid (ABA)

Prepare a 100 μM solution in sterile dH_2O.

Store in a light sensitive container at 4°C. Do not keep longer than 14 days.

B.14.2. Ampicillin

Prepare a 100 mg/ml stock in sterile dH_2O

Filter sterilise, aliquot and store at -20°C.

B.14.3. Chloroform-isoamylalcohol

Mix at a ratio of 24:1

B.14.4. DEPC (Diethylpyrocarbonate)

Prepare dH_2O with DEPC to a final concentration of 0.01% and stand overnight in the fumehood. Autoclave.

Buffers used for RNA manipulations can be treated in the same manner or they can be prepared with DEPC-treated dH_2O.

B.14.5. EDTA

0.5 M EDTA

approximately 2% NaOH

Add EDTA to 400 ml dH_2O. Slowly add NaOH until pH reaches 8.0 and EDTA dissolves. Make volume up to 500 ml with dH_2O, autoclave.

B.14.6. Ethanol 70%

70 ml absolute ethanol

30 ml sterile dH_2O

Store at –20°C

B.14.7. Ethidium bromide

Make a 10 mg/ml stock in sterile dH_2O

Store in a light-tight container.

B.14.8. IPTG (isopropyl-β-D-thiogalactopyranoside)

Prepare a 0.1 M solution in 50 ml dH_2O

Filter sterilise and store at 4°C

B.14.9. 10x MOPS

200 mM MOPS

50 mM NaAC

10 mM EDTA

Make up with RNase-free ingredients and water. Adjust pH to 7.0 with glacial acetic acid (RNase free).

B.14.10. NaCl

Prepare a 150 mM NaCl solution in dH_2O

Autoclave

B.14.11. Phenol (equilibrated to pH 7.6)

500 g commercial phenol

0.6 g 8-hydroxyquinoline

7.5 ml of 2N NaOH

6 ml of a 1M stock of Tris-HCl pH 7.6

Add all of the above (except phenol) to a bottle of phenol with 130ml of water and store overnight at room temperature.

B.14.12. PMSF (Phenyl methane sulfonyl flouride)

Prepare a 100 mM solution in isopropanal

Aliquot and store at –20°C

B.14.13. RNAse A (10 mg/ml)

Dissolve pancreatic RNase A at a concentration of 10 mg/ml in 10 mM Tris-HCl pH 7.5, 15 mM NaCl. Heat up to 100°C for 15 minutes. Allow it to cool slowly to room temperature. Dispense into aliquots and store at –20°C.

B.14.14. Sample buffer

0.25% Bromophenol blue

40% Sucrose

20 mM EDTA

B.14.15. SDS (Sodium dodecyl sulphate)

Make up 20% SDS w/v in sterile dH_2O

Heat to 80°C to dissolve

B.14.16. Tris-Borate Buffer (TBE) 5X

450 mM Tris base

450 mM Boric acid

10 M EDTA

Make up to 1 L with dH_2O and autoclave

Dilute to 1X with sterile dH_2O.

B.14.17. Tris-EDTA (TE) buffer

10 mM Tris-HCl pH 7.6

1 mM EDTA pH 8.0

Make up to 100 ml with dH_2O

Autoclave.

B.14.18. Tris-HCl

1 M Tris base

Dissolve Tris in water, adjust to desired pH with HCl, make volume up to 100 ml with dH_2O

Autoclave.

B.14.19. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside)

Prepare a 0.5 mg/ml solution in N,N`-dimethyl-formamide.

Cover with aluminum foil and store at –20°C

B.15. Media

B15.1. Luria Broth

1.0% Tyrptone

0.5% Yeast extract

0.5% NaCl

Make up in dH_2O

Autoclave

B.15.1.1. Luria Agar

Add 15 g bacterial agar per litre of LB

Autoclave

B.15.2. Ψ Broth

2% Tryptone

05% Yeast Extract

0.4% $MgSO_4.7H_2O$

10 mM KCl

Make up in dH_2O and autoclave

B.16. Calculations

B.16.1. Relative water content

RWC=((Fresh Weight-Dry Weight)/Dry Weight))/((Hydrated Weight-Dry Weight)/Dry Weight)) X 100. For each measurement the midsections of two leaves were used. The fresh weight refers to the weight of the leaf immediately after excision, the hydrated weight was determined after floating the leaf in water for 1 hour and the dry weight was obtained after drying the leaf at 100°C for 24 hours.

B.16.2. Water potential

The midsections of two leaves were placed in an Aqualab (Decagon Devices). The machine was allowed to calibrate for a minimum of one hour after which the output was monitored until a steady reading was obtained. This value was used in the following formula to obtain the water potential of the leaf sample: WP=RT/Mw x ln(aw); where T=(273.15 + ambient temperature), R is the constant 8.314, Mw (molecular weight of water) is 18 and ln(aw) is the natural log of the water activity as measured by the machine.

APPENDIX C

PRIMERS AND PLASMID DIAGRAMS

C.1. Primers

C.1.1. Universal primer
5`-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3`

C.1.2. Hsp90Rev3`A
5`-GCGTGAGTTACAACTCATCCTTGGCGCTG-3`

C.1.3. Hsp90atg
5`-AGATGAGGAATTGGTCGATCC-3`

C.1.4. Rev5`C
5`-CGCCTGACATATAGCTTCAAG-3`

C.1.5. Hsp90atg2
5`-CGATGAGGAATTGGTCGATCC-3`

C.1.6. RTFor
5`-TTGAAACCACCAAGTCTGATG-3`

C.1.7. M13 Forward primer
5`-GTAAAACGACGGCCAGT-3`

C.1.8. M13 Reverse primer
5`-CTACCAGTATCGACAA-3`

C.1.9. Rev5'D
5`-GACTTCAACATAGTCAGAAAC-3`

C.1.10. Rev5`C
5`-CGCCTGACATATAGCTTCAAG-3`

C.1.11. Hsp90F
5`-CGGCACGAGATAAAGCTTGG-3`

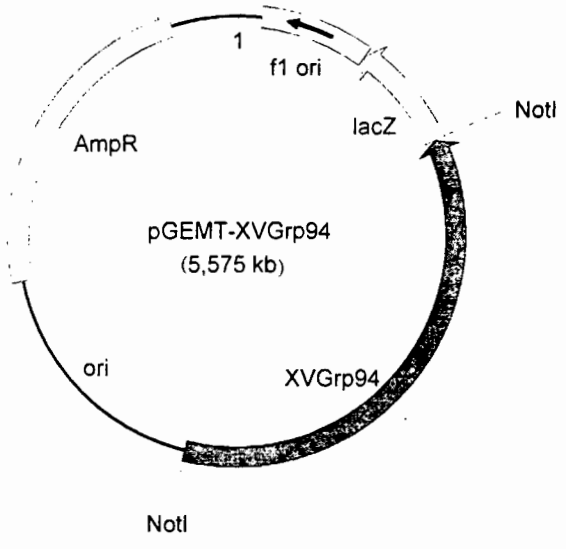
C.1.12. HspRev5`A
5`-GGCGATTTCTATTTTGTGCATC-3`

C.1.13. Hsp90_5`A
5`-CCGTGAAGATTAGCAACCG-3`

C.1.15. HomHspF
5`-GGGCAATTCGGTGTTGGTTTCTATTCAG-3`

C.2. Plasmid diagrams

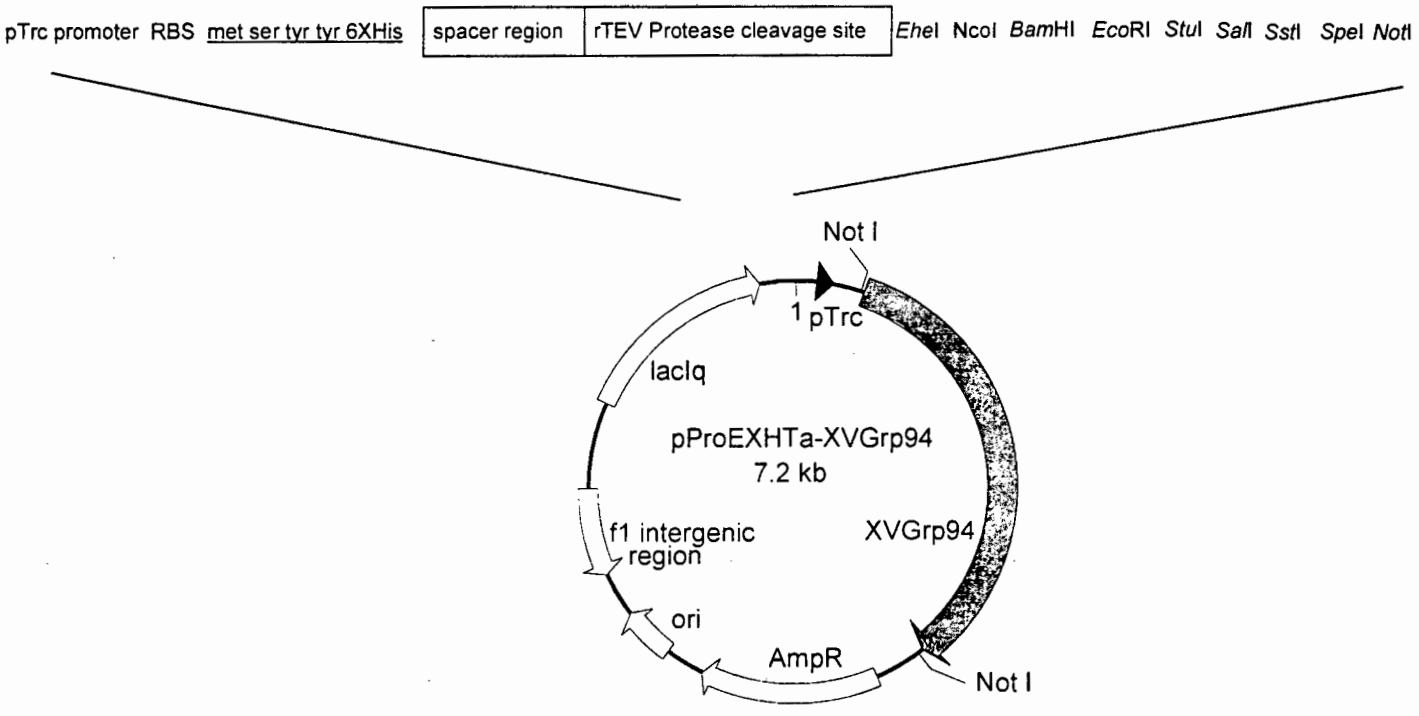
C.2.1. pGEMT-XVGrp94



C.2.1a pGEMT-XVGrp94(ORF)

As for pGEMT-XVGrp94 (C.2.1) except that the XVGrp94 insert is approximately 100 bp shorter at the 5` terminus.

C.2.2. pProEX HTa-XVGrp94



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